Tuveson Laboratory Murine and Human Organoid Protocols

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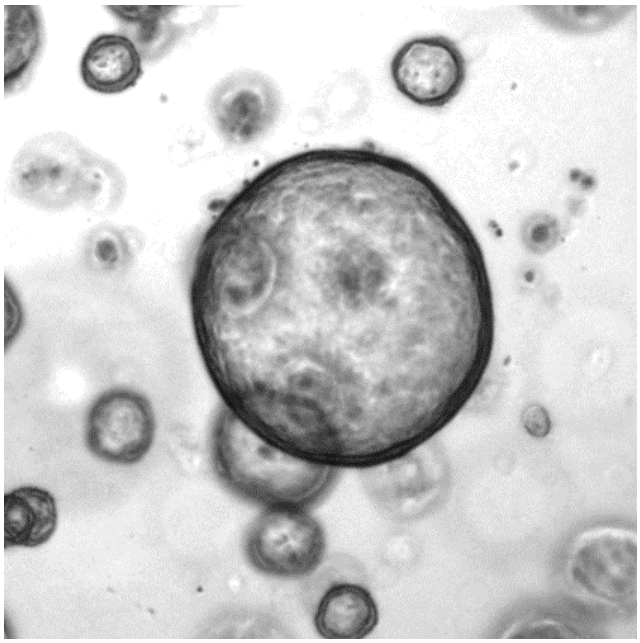
Based on methods described in:

Huch M et al. 2013 Unlimited in vitro expansion of adult bi-potent pancreas progenitors through the Lgr5/R-spondin axis. EMBO J. 32: 2708-21.

Boj S et al. 2015 Organoid models of human and mouse ductal pancreatic cancer. Cell 160: 324-38.



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Protocol for Isolation of Organoids from Normal or PanIN Mouse Pancreas

(edits by LB 12.04.2013; based on MSS/BC 5.16.2013 N/P Protocol and CH/CC/YP 9.10.2013 T Protocol)

Notes

- This protocol is for making normal organoids or organoids from mice containing PanINs (e.g. KC mice). To make organoids from tumor-bearing mice, see the "Protocol for Isolation of T and M Organoids from Mouse Pancreatic Tumors and Metastases" on page 8.
- If possible, schedule at least 2 people to be involved:
    - Person 1 will handle the pancreas dissection, will conduct the necropsy if needed, will clean the necropsy room, and then will pick ducts in the microscope.
    - Person 2 will prepare the digestion materials and the tissue culture hood, will set up the digestion, and will eventually assist in duct picking and plating.
- Person 1 should start prepping the digestion solution and tissue culture hood about 1 hour before the mouse is to be sacrificed.
- Overall protocol time (including preparation and plating) is about 4 hours.
- Organoids grow under same conditions as 2D cell lines (37^{o}C, 20% O_2, 5% CO_2)

A. Isolation of Mouse Pancreas

You will need the following (have ready in the necropsy room):

Petri dish (not tissue culture plate)
Dissection tools
Dissection board
Marker
Hot bead sterilizer for dissection tools
Eppendorf tube for mouse tail
Digital scale
Strong scissors for decapitation
Paper towels and blue pad
CO_2 chamber with CO_2 hookup and regulator
Mouse

If PanIN mouse, you will also need:

10% Neutral Buffered Formalin (NBF) for histology
Pencil to mark histology cassettes
Histology cassettes
Histology worksheet

Procedure:

1. Sterilize dissection tools in hot bead sterilizer.
2. Prepare your work area:
    a. Put down a clean blue pad.
    b. Tack a clean paper towel to your dissection board
    c. Set out paper towels for dissection tools and blotting mouse blood.
    d. Separate dissection tools and scissors into three sets, (1) scissors for the mouse decapitation, (2) dissection tools for cutting through the mouse skin and peritoneum, and (3) dissection tools for removing the pancreas.
    e. Place a paper towel in CO_2 chamber.
3. If PanIN mouse, label cassettes for histology with pencil and begin filling in histology worksheet. (Typically, we don't do histology on wild-type mice.)
4. Label a clean Petri dish, and tare the digital scale with it.
5. Record information about mouse (strain, name, date of birth).
6. Sacrifice mouse in CO_2 chamber:
    a. Turn the CO_2 on very slowly to keep the mouse as comfortable as possible.
    b. Watch the mouse the entire time.
    c. The mouse should go from active, to inactive but breathing normally, to making quick gasps, to making heaving gasps/spasms with longer pauses in between.

    d. After 2 or 3 heaving gasps remove mouse from chamber.
    e. It is important to work quickly, because the viability of the pancreas will decrease with time. After opening up the mouse you will notice that with time, the pancreas turns from healthy pink to unhealthy gray.
7. While the mouse's heart is still beating, position strong scissors between head and neck, and cut off the head.
8. Blot the mouse's neck onto paper towels until blood flow slows.
9. Spray mouse with ethanol.
10. Skin the mouse.
    a. Locate the mouse's sternum (where the chest bone sticks out).
    b. Pull the fur up here, and make a sizeable cut through the skin (but not through the peritoneum).
    c. Cut skin towards back side of mouse.
    d. With two fingers of each hand, pinch the skin on both sides of the opening and pull the skin towards the head and tail until entire midsection is exposed.
11. Cut open the peritoneum near the genitals, and cut to expose the intestines and pancreas.
12. Switch to clean dissection tools!
13. Remove the pancreas, while being careful not to rupture the intestines.
    a. To locate the pancreas, either locate the spleen and start there while working down toward the intestines, or carefully pull out the intestines, working your way up from the cecum to the stomach.
    b. If intestines are ruptured, your organoids may be contaminated! If possible, discard this pancreas and start over with a new mouse.
14. Place pancreas in the clean, tared Petri dish.
15. Place the Petri dish on the digital scale, and record the weight of the pancreas.
16. If mouse had PanIN, cut a piece of pancreas, place in a labeled histology cassette, and place cassette in a container of 10% NBF to fix for histology.
17. Pass the Petri dish containing the pancreas to the person who will digest pancreas. The pancreas should be brought immediately to the Tissue Culture Room to start digestion procedure described in Part B of this protocol.
    a. If you are working alone, go immediately to a tissue culture hood and begin mincing the pancreas as described in Part B of this protocol. Once the pancreas is incubating in Mouse Digestion Media, return to the necropsy room to finish necropsy and clean room as described below.
    b. Waiting too long between harvesting the pancreas and getting it into digestion can cause the pancreas to over-digest itself and organoid isolation to fail.
18. If mouse had PanIN, continue with full necropsy and fill cassettes to send to histology.
19. Cut tail off of mouse and save for confirmation genotyping.
20. Clean up necropsy room.
    a. At Cold Spring Harbor Laboratory, used mouse cages should be covered with trash bag or hair nets and returned to back room of mouse facility.
    b. Mouse carcass should be wrapped in a glove and placed in the bucket at the bottom of the chest freezer in the back room of the mouse facility.

B. Pancreas Digestion and Duct Picking

You will need:

Ice bucket with ice
Dispase II
Collagenase Crude Type XI (from *Clostridium histolyticam)*
Thermomixer or incubated rocker/rotator set to 37°C
15 mL Falcon Tubes
#10 Scalpels
Petri Dishes (ones with grids if possible)
Extra Styrofoam tube holders or boxes (for armrests for pickers)
Microscope for each duct picker
Matrigel on ice
48-well plate
37°C water bath
Refrigerated 15/50 mL tube centrifuge
Mouse Wash Media on ice
10 mg/mL DNase I
Sterile glass bottle
Sterile 10 and 25 mL pipettes
Sterile glass pipettes and aspirator
Sterile P20, P200, P1000 Pipetmen with tips inside TC hood
P10 or P20 Pipetmen with tips for each duct picker
Mouse Complete Feeding Media with RhoK Inhibitor (Y-27632)
Hot water bottle
Marker
Tissue culture incubator
Timer
Microscope

Procedure:

1. Before starting the organoid preparation, place Mouse Complete Feeding Media in the 37°C water bath to ensure it comes to at least room temperature by the time the organoids are ready to be fed. (Mouse Complete Feeding Media can be made up to 2 weeks in advance).
2. Prepare Mouse Wash Media and keep on ice:
   Add 5 mL 100% FBS and 5 mL 100X Penicillin/Streptomycin to 500 mL DMEM
   Mouse Wash Media can be stored for 1 month at 4°C, and used for additional isolations.
   ~300 mL Mouse Wash Media are needed per mouse.
3. Prepare Mouse Digestion Media (0.125 mg/mL Collagenase Crude Type XI (Collagenase from *Clostridium histolyticam*), and 0.125 mg/mL Dispase II in DMEM/1% FBS/1% Pen-Strept) in a sterile glass bottle and allow it to warm to room temperature.
   a. Weigh 25 mg Collagenase Crude Type XI and 25 mg Dispase II into a 50 mL conical tube (outside Tissue Culture hood)
   b. Inside Tissue Culture Hood: add 50 mL Mouse Wash Media to 50 mL tube containing enzymes, mix and pour into glass bottle.
   c. Repeat 3 more times with 50 mL Mouse Wash Media to bring final volume to 200 mL.
4. Place a 48-well tissue culture plate on top of a pre-warmed hot water bottle in a 37°C tissue culture incubator.
5. Thaw aliquots of 10 mg/mL DNase I and Matrigel on ice.
6. Prepare a 15 mL Falcon tube with 5 mL Mouse Wash Media to receive the minced pancreas, and place tube on ice.
7. Add 2 mL cold Mouse Wash Media to Petri Dish with pancreas.
8. Mince pancreas in the Mouse Wash Media in Petri dish with #10 scalpels, keeping the dish on ice while mincing. Be careful not to over-mince! Just get pancreas broken up into ~1-2 mm size pieces.
9. Transfer minced pancreas to 15 mL Falcon Tube containing Mouse Wash Media.
10. Use 5 mL Mouse Wash Media to rinse Petri dish and get all pancreas pieces into Falcon Tube.
11. Let tube stand at room temperature for 1-2 minutes. If present, fat should rise to top and float on surface, while pancreas material should sink.
12. Aspirate off fat and most of Mouse Wash Media, leaving ~1 mL Mouse Wash Media with pancreas pieces in bottom of tube. Be careful not to aspirate pancreas pieces.

13. Add 10 mL Mouse Digestion Media to pancreas.
14. Incubate at 37°C for 20 minutes, with gentle agitation.
    If you are working alone, continue with mouse necropsy during this incubation.
15. Prepare a 15 mL Falcon Tube with 5-10 mL Mouse Wash Media for each duct picker.
16. After 20 minutes, remove tube with pancreas digest from 37°C.
17. Pipet up and down with 10 mL pipette to break up chunks.
18. Let tube stand ~1 minute to allow pancreas pieces settle by gravity.
19. Transfer 8.5 mL of supernatant to a clean Petri dish that contains 10 mL Mouse Wash Media. This is "Wash 1."
20. Give Petri dish containing wash to picker to check in microscope for the presence of ducts and if present, pick ducts.
    a. If you are working alone, proceed immediately to step 21, and then pick ducts during 10 minute incubations.
    b. To pick ducts, look under the microscope, using the 4X or 10X objective. With the phase contrast on, in the 10X objective, ducts appear like small chains of cells, are smaller than blood vessels, and often have lariat or cross shapes. For more information, refer to Figure 1.
    c. Use a P10 or P20 set to 8-10 µL. Push Pipetman plunger to first stop, hover pipette tip near a duct. Release Pipetman plunger slightly to suck up duct. You should see the duct go into the pipette tip. Keep finding and sucking up ducts until you have released the plunger all the way. Then dispel ducts into 15 mL Falcon tube with Mouse Wash Media.
    d. Most people prefer to use their dominant hand for picking, and to position a box or Styrofoam tube rack under their elbow to help stabilize it.
    e. Avoid picking acinar cells (refer to Figure 1), because in large numbers, these will prevent organoids formation. Avoid picking blood vessels, which are similar to ducts, but are a bit larger in size (thicker diameter) with a visible, hollow lumen. However, **when in doubt, pick it** – small numbers of acinar or blood vessels will still permit organoid formation.
    f. For wild-type mice, ducts usually appear at the 3rd-4th wash. For PanIN and tumor mice, ducts may take longer to appear, and may start showing up as late as the 7th wash.
    g. Try to get as many ducts as possible – at least 100 or more is ideal.
    h. For PanIN mice, pick both normal and abnormal ducts.
    i. If there are too many cells around ducts for clean picks, it may help to dilute washes 1:2 with additional Mouse Wash Media and split into 2 Petri dishes.
    j. The entire picking process happens outside the tissue culture hood with open tubes and open Petri dishes. For us, there have not been contamination issues.
21. Add 8.5 mL more Mouse Digestion Media to Falcon tube with pancreas and incubate at 37°C for 10 minutes with gentle agitation.
22. After 10 minutes, remove tube from 37°C and repeat steps 17-21 to generate "Wash 2." Repeat to generate "Wash 3," "Wash 4," etc.
    a. Starting at the 4th wash (or earlier if cells start to burst and digestion looks viscous before the 4th wash) add 10 µL 10 mg/mL DNase to the Mouse Digestion Media at each new incubation.
    b. Continue making new washes until no ducts are visible in the washes anymore (usually at least 8 washes, for PanIN could be up to 12)
23. Once ducts are picked into 15 mL Falcon Tube(s), spin tube(s) in the clinical centrifuge at 850 rpm (145 RCF) for 5 minutes at 4°C.
    Note: Pellet will be very small, or may not be visible.

24. If you have more than 1x 15 mL Falcon tube with ducts from the same mouse, aspirate off most of the media and use Wash Media to pool all ducts into 1x 15 mL Falcon tube. Then spin again at 850 rpm (145 RCF) for 5 minutes at 4^{o}C.
25. Remove as much media as possible: aspirate down to ~200 µL and use Pipetman to carefully remove the rest. (Residual media will dilute the Matrigel and may prevent Matrigel domes from solidifying, so try to remove as much media as possible without sucking up your pellet.)
26. Resuspend pellet in Matrigel and plate 25 µL Matrigel domes in a pre-warmed 48-well plate on top of a pre-warmed hot water bottle.
    a. Typically, we resuspend in 125 µL Matrigel and make 5 x 25 µL wells in a 48 well plate, but may vary depending on pellet size.
    b. More information about plating Matrigel domes can be found in the "Protocol for Passaging Mouse Organoids" on page 13.
27. While keeping plate on hot water bottle, carefully move plate and bottle into 37^{o}C incubator. Keep at 37^{o}C 5-15 minutes so Matrigel can harden.
28. Add Rho Kinase Inhibitor Y-27632 to the Mouse Complete Feeding Media. You will need 250 µL media per well plated. The 10.5 mM stock of Y-27632 is a 1000X stock.
29. After 5-15 minutes, remove 48-well plate on hot water bottle, and add 250 µL of warm/room temperature Mouse Complete Feeding Media with Rho Kinase Inhibitor to each well. Once organoids are fed, plate no longer needs to sit on hot water bottle.
30. Check plate in microscope – ducts should be visible in the Matrigel.
31. Return plate and hot water bottle to incubator.

C. Post-Isolation

1. Within 1-2 days, organoids should start to bud out of ducts. (Refer to Figure 2.)
2. If mouse has PanIN, make sure to finish preparing cassettes for histology. In the Tuveson Laboratory, we incubate histology cassettes overnight in 10% NBF. After 16-24 hours, carefully pour the 10% NBF off of the cassettes and add 70% ethanol to cover the cassettes. Cassettes should be brought to histology within 1-2 days of placing them in 70% ethanol. P and T organoids must have PanIN or Tumor histology confirmed on tissue section.
3. Change media after 2-4 days, depending on how fast organoids grow. Rho Kinase Inhibitor is not needed in media after the initial isolation.
4. Let organoids grow for ~ 1 week before passaging. For first passage after isolation:
    a. Do not scrape bottom of plate under Matrigel; leave some Matrigel on bottom to avoid contaminating next passage with fibroblasts that have attached to the plate.
    b. Harvest all wells, pool, and freeze down the equivalent of one well into 2 cryovials as passage 0.5 Expand the rest of the organoids 1:4 or 1:8 (depending on density) in unused wells of the same plate or a new plate.
    c. See the "Protocol for Passaging Mouse Organoids" on page 13 and the "Protocol for Freezing Mouse Organoids" on page 16 for more information on how to passage and freeze organoids.
    d. The Tuveson Lab convention is that organoids initially isolated from a mouse are passage 0, and that when organoids are frozen they are given a passage number halfway between the passage that they came from and the passage that they will be upon thawing.

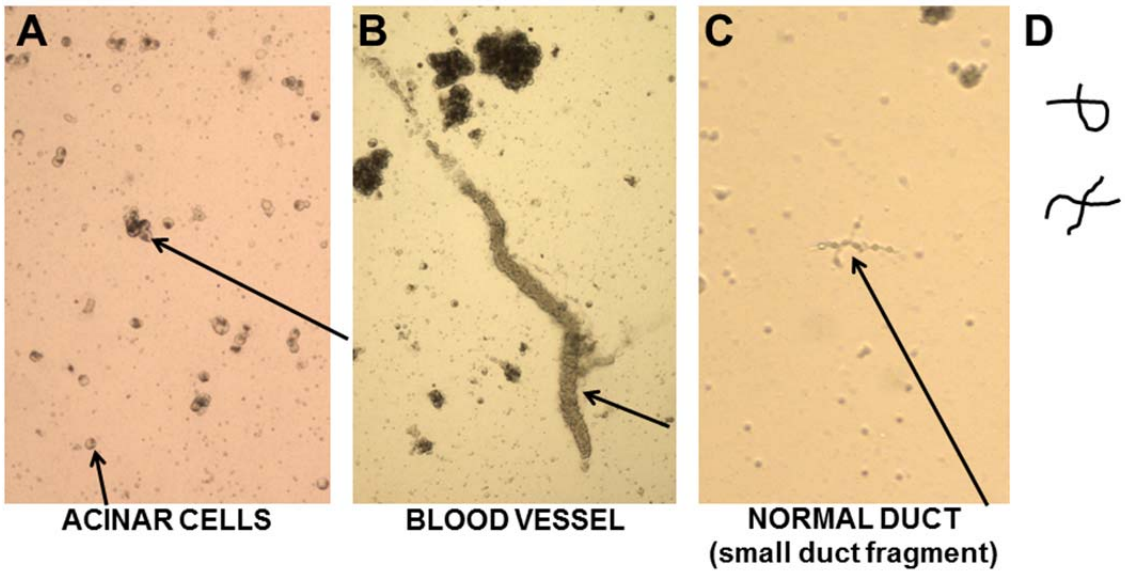

Figure 1. High magnification images of cell types found in pancreas digestion viewed with phase contrast microscope.

A. Arrows denote acinar cells, which are large cells, may come in clumps, and are often more refractile. **B.** Arrow indicates a blood vessel, which is generally larger than most ducts. Unlike most ducts, the lumen of the blood vessel is often visible, and red blood cells may be visible inside the vessel. **C.** Arrow indicates a typical small pancreatic duct fragment. Note that these images were taken with a higher magnification objective, and ducts will look smaller in the 4x objective. **D.** Small duct fragments often have lariat or cross structures, as diagramed here.

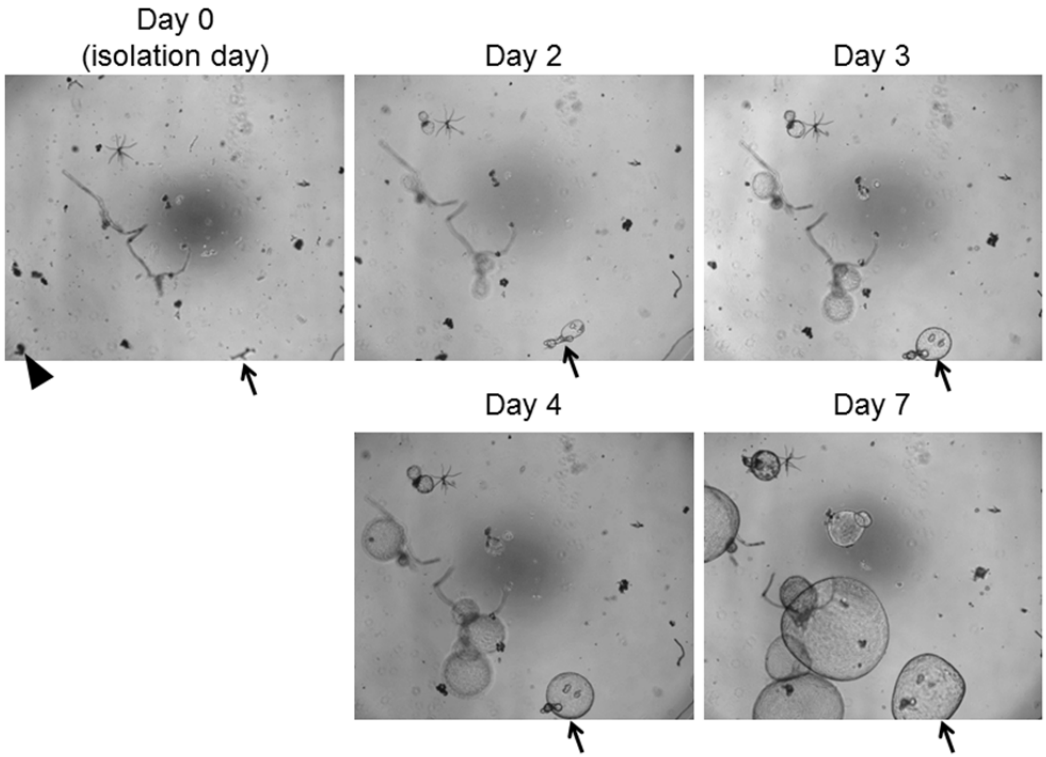

Figure 2. Murine normal organoid formation following duct picking.

Images of a single well immediately following duct isolation and plating, as well as 2, 3, 4, and 7 days post-isolation. Arrowhead indicates an acinar cell, which looks dark in the Matrigel. Arrow indicates a typical small duct, with branched structure, and the organoid that grows out from it.

Protocol for Isolation of T and M Organoids from Mouse Pancreatic Tumors and Metastases

You will need:

(have ready in the necropsy room)

- Petri dish (not tissue culture plate)
- Dissection tools
- Dissection board
- Marker
- Hot bead sterilizer for dissection tools
- Eppendorf tube for mouse tail
- 10% Neutral Buffered Formalin (NBF) for histology
- Pencil to mark histology cassettes
- 15 mL Falcon Tubes with 10 mL Mouse Wash Media on ice
- Digital scale
- Strong scissors for decapitation
- Paper towels and blue pad
- CO_2 chamber with CO_2 hookup and regulator
- Mouse with tumor
- #10 Scalpels
- Histology cassettes
- Histology worksheet
- Ice bucket with ice

(have ready in the Tissue Culture facility)

- Ice bucket with ice
- Dispase II
- Collagenase Crude Type XI (from *Clostridium histolyticam)*
- Thermomixer or incubated rocker/rotator set to 37°C
- Refrigerated 15/50 mL tube centrifuge
- 15 mL Falcon Tubes
- TrypLE Express
- Petri Dishes (ones with grids if possible)
- Extra Styrofoam tube holders or boxes (for armrests for pickers)
- Microscope for each duct picker
- Matrigel on ice
- 48-well plate
- 37°C water bath
- (for generating 2D line) 2D Cell Culture Media
- (for blood collection) Refrigerated microfuge
- Mouse Wash Media on ice
- 10 mg/mL DNase I
- Sterile glass bottle
- Sterile 10 and 25 mL pipettes
- Timer
- Sterile glass pipettes and aspirator
- Sterile P20, P200, P1000 Pipetmen with tips inside TC hood
- P10 or P20 Pipetmen with tips for each duct picker
- Mouse Complete Feeding Media with RhoK Inhibitor (Y-27632)
- Hot water bottle
- Marker
- Tissue culture incubator
- Splitting Media (+++)
- (for blood collection) Animal lancet
- (for blood collection) Heparin tube

Procedure:

1. Prepare necropsy area and histology cassettes as described in the “Protocol for Isolation of Organoids from Normal or PanIN Mouse Pancreas”, on page 2.
2. Prepare Mouse Wash and Mouse Digestion Media as described in steps 2 and 3 in the the “Protocol for Isolation of Organoids from Normal or PanIN Mouse Pancreas”, on page 4.
3. Optional: before sacrificing mouse, using an animal lancet, perform a submandibular bleed on mouse. Collect blood in a tube containing heparin. Spin tube for 2 minutes at 10,000 rpm at 4°C in a microfuge, and pipette supernatant (plasma) into new tube. Flash freeze plasma and store at -80°C.
4. Rather than dissecting out whole pancreas, dissect out tumor and place on a Petri dish on ice.
5. In the Petri dish, cut a central piece of the tumor and place in a labeled cassette for histology. Place cassette in a container containing 10% NBF.
6. Mince the tumor in a Petri dish on ice, and move to a 15 mL Falcon tube containing Mouse Wash Media.

7. Prepare the rest of the mouse organs for histology. Check carefully for metastases (mets), and if a met is located, send a portion of it for histology, and use a portion of it to make an “M” organoid (see step 23 below).
8. Bring the tubes of tumor (and mets) on ice to the tissue culture hood.
9. Allow the tumor pieces to settle, and aspirate the fat as described in steps 11-12 in Part B of the “Protocol for Isolation of Organoids from Normal or PanIN Mouse Pancreas”, on page 4.
10. Add 10 mL Mouse Digestion Media and incubate on a rocker at 37^{o}C for 8-18 hours.
11. Remove digested tumor from 37^{o}C.
12. Pipet up and down with 10 mL pipette to break up chunks.
13. Let pancreas settle by gravity.
14. Transfer 8.5 mL of supernatant to new tube.
15. Spin this tube at 1000 rpm (200 RCF) for 5 minutes at 4^{o}C.
16. Aspirate the supernatant.
17. Resuspend the pellet in 1 mL TrypLE Express and 1 µL 10 mg/mL DNase I.
18. Incubate at 37^{o}C in with gentle agitation for 10 min.
19. Add 10 mL Splitting Media (+++) to TrypLE-digested pancreas.
20. To make a 2D cell line, take 1 mL of pancreas digest and add to 10 cm tissue culture dish containing 9 mL 2D Cell Culture Media.
    Once 2D cell line is established, 2D Cell Culture Media with 5% FBS can be used to slow growth.
21. Spin remaining pancreas digest at 1000 rpm (200 RCF) for 5 min at 4^{o}C.
22. Aspirate media, resuspend pellet in Matrigel, and plate cells as described in steps 25-29 in Part B of the “Protocol for Isolation of Organoids from Normal or PanIN Mouse Pancreas”, on page 6. (Be sure to include Rho Kinase Inhibitor in the Mouse Complete Feeding Media).
23. For small mets, incubate for 1 hour in Mouse Digestion Media at 37^{o}C with gentle agitation, and follow steps 11-22 of this protocol.
24. Over the next 2-3 days, small spheres should begin to emerge among the cells plated (Figure 3).
25. At the first passage, there may be fibroblasts at the bottom of the wells. When passaging the first 1-2 times, avoid scraping all the way to the bottom of the well with your pipette tip, to avoid passaging the fibroblasts.

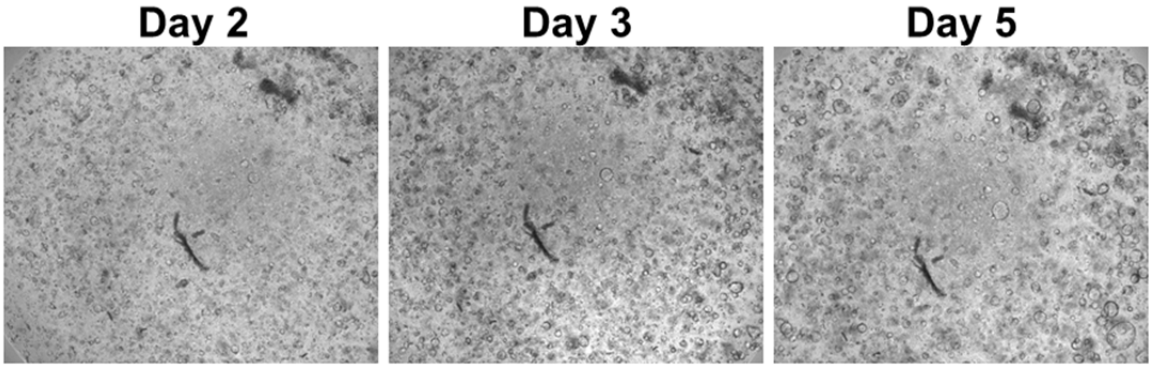

Figure 3. Formation of murine tumor organoids following isolation.
Images of a single well 2, 3, and 5 days following tumor digestion and plating. Small spheres are visible as soon as 2 days following plating.

Media Recipes for Murine Organoid Protocols

Please see page 54 for Suppliers and Catalog Numbers

MEDIA RECIPES

Mouse Wash Media

500 mL	DMEM
5 mL	100X Penicillin/Streptomycin (f.c. 1x)
5 mL	100% FBS (f.c. 1%)

Used for mincing pancreas, fat aspiration, diluting washes for picking, and for tubes that receive picked ducts.
Need ~400 mL per mouse for N or P organoid (200 for Mouse Digestion Media + ~200 for washes).
Need ~200 mL for T or M organoid isolation (200 for Mouse Digestion Media).
Can be prepared in advance and stored at 4°C for up to 1 month.
Unused Mouse Wash Media can be stored at 4°C and used for next isolation.

Mouse Digestion Media

200 mL	Mouse Wash Media
25 mg	Collagenase Crude Type XI (f.c. 0.125 mg/mL)
25 mg	Dispase (f.c. 0.125 mg/mL)

Used for digesting pancreas or tumor.
Make this fresh before use.
You need 100 mL per mouse for N or P organoid, and 10 mL for T or M organoid, but it is difficult to make less than 200 mL at a time.
Typically, we make as follows:

a. Take Collagenase out of -20°C freezer and Dispase out of fridge, and allow containers to come to room temperature.
b. Weigh Collagenase and Dispase carefully and pour each into 50 mL Falcon Tube. (Technically, this step is not done in sterile conditions, but seems to work ok.)
c. In Tissue Culture Hood, add 50 mL of DM to to Falcon Tube with enzymes and invert to get enzymes into solution
d. Pour Mouse Wash Media with enzymes into a sterile glass bottle
e. Add 50 mL more Mouse Wash Media to 50 mL Falcon Tube to rinse tube, and pour this into sterile bottle. Repeat 2 more times to bring volume to 200 mL.

Splitting Media (+++)

485 mL	Advanced DMEM/F-12
5 mL	1M HEPES
5 mL	Penicillin/Streptomycin
5 mL	GlutaMAX

Can be made in advance and stored at 4°C for up to 2 months.

Mouse 2D Cell Culture Media

445 mL	DMEM
50 mL	FBS (f.c. 10%)
5 mL	100X Penicillin/Streptomycin

Can make in advance and store at 4°C.
Once cell lines are established, may want to switch to 2D media with 5% FBS instead of 10% to slow cell growth.

Mouse Complete Feeding Media with Rho Kinase Inhibitor

	Advanced DMEM/F-12
1X	1M HEPES (comes as 100X stock)
1X	Penicillin/Streptomycin (comes as 100X stock)
1X	GlutaMAX Supplement (comes as 100X stock)
0.5 µM (0.21 µg/mL)	A83-01 (TGF Beta Inhibitor)
0.05 µg/mL	mEGF
0.1 µg/mL	FGF-10
0.01 µM (0.021 µg/mL)	Gastrin I
0.1 µg/mL	mNoggin
1.25 mM (0.2 mg/mL)	N-acetylcysteine
10 mM (1.22 mg/mL)	Nicotinamide
1X	B27 supplement
10%	R-spondin-conditioned media
10.5 µM (3.38 µg/mL)	Y-27632 (Rho Kinase Inhibitor)*

Make up to 2 weeks in advance and store at 4^{o}C.

See “Protocol for Making Murine Splitting and Feeding Medias” on page 12 and “More Information About Feeding Media Components” on page 46 for further information about the Organoid Media and components.

*The Y-27632/Rho Kinase Inhibitor helps cells cope with stress, and is only added to the Mouse Complete Feeding Media when organoids are isolated for the first time, when organoids are thawed, or when organoids are dissociated down to single cells.

Protocol for Making Murine Splitting and Feeding Medias

Notes:
Because some of the components of the Mouse Complete Feeding Media may not be stable in solution, we try to make the media no more than 2 weeks before using it.
The base for the Mouse Complete Feeding Media is Splitting Media (+++), which can be made in advance and stored at 4^{o}C, and is good for at least 1 month.
Mouse Complete Feeding Media can be made up to 2 weeks in advance and stored at 4^{o}C until use.

Splitting Media (+++) Recipe

VOLUME	STOCK CONCENTRATION	STOCK
500 mL		Advanced DMEM/F-12
5 mL	100X (1M)	1M HEPES
5 mL	100X	Penicillin/Streptomycin
5 mL	100X	GlutaMAX

Mouse Complete Feeding Media Recipe

VOLUME	STOCK CONCENTRATION	STOCK CONCENTRATION	STOCK	FINAL CONCENTRATION
18 mL			Splitting Media (+++)	
20 µL	1000X	0.5 mM (0.21 mg/mL)*	A83-01*	0.5 µM (0.21 µg/mL)
20 µL	1000X	50 µg/mL*	mEGF*	0.05 µg/mL
20 µL	1000X	0.1 mg/mL*	FGF-10*	0.1 µg/mL
20 µL	1000X	10 µM (0.021 mg/mL)*	Gastrin I*	0.01 µM (0.021 µg/mL)
20 µL	1000X	100 µg/mL	mNoggin	0.1 µg/mL
50 µL	400X	500 mM (81.5 mg/mL)	N-acetylcysteine	1.25 mM (0.2 mg/mL)
200 µL	100X	1M (122 mg/mL)	Nicotinamide	10 mM (1.22 mg/mL)
2 mL	10X		R-Spondin-conditioned media	
400 µL	50X		B27 supplement (50x)	1X
20 µL	1000X**	10.5 mM (3.4 mg/mL)	Y-27632**	10.5 µM (3.38 µg/mL)

* 1000X working stocks of A83-01, mEGF, FGF-10, and Gastrin I are prepared by diluting 10,000X frozen stocks – see “Solutions and Key Reagents” for more information.
** Y-27632 (Rho Kinase Inhibitor) is only necessary when organoids are first prepared, when organoids are thawed, or when organoids are dissociated to single cells.

Protocol for Passaging Mouse Organoids

Notes:
We culture organoids in a dome of Matrigel bathed in a liquid media. Because organoids are suspended in the Matrigel, they never touch the plastic of a tissue culture dish like a 2-dimensional culture would. Matrigel is a gelatinous protein-rich substance secreted by EHS mouse sarcoma cells, and sold by BD Biosciences. Because of this, there is some variation between Matrigel content, protein content, and stiffness from lot to lot. Individual lots need to be tested for organoid culture. In practice, we use lots with protein content between 8-9 mg/mL.
Matrigel is frozen at -20°C, liquid at 0°C, and begins to harden above 0°C. Always keep Matrigel aliquots on ice when working with it.
Typical split ratios are between 1:4 and 1:8, depending on organoid confluency. Organoids are typically ready to passage again 3-8 days after passaging.

You will need:

24- or 48- well tissue culture plate	Hot-water bottle
37°C tissue culture incubator	Mouse Complete Feeding Media at 37°C
Splitting Media (+++) on ice	Ice bucket with ice
15 mL Falcon tube on ice	Matrigel aliquot on ice
P1000 and P200 Pipetmen and sterile tips	Sterile glass pipettes and aspirator
Fire-polished glass pipette or needle w/ syringe	Refrigerated 15/50 mL tube centrifuge

Procedure:

1. Up to 2 weeks before you begin, make Mouse Complete Feeding Media. Up to 4 weeks in advance, make Splitting Media (+++).
2. Just before starting, place Mouse Complete Feeding Media in 37°C water bath to warm up. Mouse Complete Feeding Media must be at least room temperature when you add it to Matrigel domes, or it will cause the Matrigel to melt.
3. Remove Matrigel aliquot and place on ice to thaw.
   An 800 µL aliquot takes ~1 hour 15 minutes to thaw. A complete Matrigel vial takes ~10-16 hours to thaw.
4. Place a 24- or 48-well tissue culture plate on top of a pre-warmed hot water bottle in a 37°C tissue culture incubator.
5. If using fire-polished pipettes to break up organoids, make these ahead of time.
6. Place Splitting Media (+++) on ice. Prepare a 15 mL Falcon tube with 8 mL Splitting Media (+++) to receive organoids and place on ice.
7. Aspirate media from organoid wells to be split.
8. Add 500 µL (for 24-well plate) or 250 µL (for 48-well plate) ice-cold Splitting Media (+++) to each well to be split, and pipette up and down and scrape the bottom of the well to dislodge the Matrigel and break it up.
9. Transfer the Splitting Media (+++) containing the broken-up Matrigel to an ice-cold 15 mL Falcon Tube containing 8 mL ice-cold Splitting Media (+++).
10. Use additional ice-cold Splitting Media (+++) to wash organoid wells, and fill Falcon Tube containing old Matrigel/organoids with Splitting Media (+++) to 10 mL.
11. Spin Falcon Tube containing old Matrigel/organoids at 850 rpm (145 RCF) for 5 min at 4°C.

12. Aspirate off media until approximately 1.5 mL media remain.
    Aspirate carefully, keeping the tip of the aspirator pipet at the top of the liquid.
13. Use fire-polished pipette to break up organoids. Alternatively, use a 22- or 23-gauge needle attached to a 5 mL syringe.
    a. If using a fire-polished pipette, attach pipette to pipette gun and pipet organoids up and down through pipette 7-12 times. Initially, you should see large particles coming up into pipette which should get smaller as you pipette them up and down.
    b. If using a needle and syringe, carefully assemble needle and syringe, remove syringe cap, and lower needle and syringe into 15 mL Falcon tube. Slowly bring up syringe plunger to take in as much media as possible. (With a 1 inch needle and 5 mL syringe, you won't be able to take in all the media, but this is ok.) While holding needle at bottom of tube, push media back out through needle. This will dislodge organoid pellet from bottle of tube. Repeat pulling media and organoids into syringe and pushing out again 6-8 times.
14. Fill 15 mL Falcon tube to 10 mL with ice-cold Splitting Media (+++).
15. If you do not plan to use all organoids for split, discard appropriate amount of organoids (or move to another tube for freezing), and refill tube to 10 mL with ice-cold Splitting Media (+++).
    a. For example, if you are splitting 1 well of a 24 well plate 1:8, and plan to make 4 new wells in a 24-well plate, discard 5 mL / 10 mL of organoids at this step.
16. Spin organoids at 850 rpm (145 RCF) for 5 minutes at 4^{o}C.
17. Carefully aspirate off as much media as possible.
    a. Get as much media as possible with aspirator and then carefully remove the rest with a P200 Pipetman.
18. Resuspend organoid pellet in Matrigel.
    a. Use 50 µL Matrigel per new well of 24-well plate or 25 µL Matrigel per new well of 48-well plate.
    b. Keep Matrigel tube and organoid tube on ice the entire time.
    c. Be very careful not to produce bubbles when pipetting Matrigel.
19. Remove new 24- or 48-well plate on top of hot water bottle from 37^{o}C tissue culture incubator and spot a 50 µL Matrigel dome for each well of a 24-well plate or a 25 µL Matrigel dome for each well of a 48-well plate.
    a. To spot Matrigel dome, keeping 15 mL Falcon tube with Matrigel and organoids on ice, draw Matrigel and organoids into Pipetman, touch tip to the center of a well on a 24-/48-well plate on top of water bottle, and push down pipette plunger while slowly pulling Pipetman up. The Matrigel and organoids should form a dome in the center of the well.
    b. Do not push Pipetman plunger to second stop. This will form bubbles.
    c. Keep tube containing organoids in Matrigel on ice throughout this procedure. This will keep Matrigel liquid and prevent it from hardening too early or inconsistently.
20. Carefully bring 24- or 48-well plate on top of hot water bottle back to 37^{o}C tissue culture incubator, and incubate at 37^{o}C for 5-15 minutes to allow Matrigel to harden.
21. Bring plate and hot water bottle back to tissue culture hood and add 500 µL (for wells in a 24-well plate) or 250 µL (for wells in a 48-well plate) of pre-warmed Mouse Complete Feeding Media to each well.
22. Check wells in phase-contrast microscope. You should see small pieces of organoids distributed throughout the Matrigel.
23. Return 24- or 48-well plate containing organoids to 37^{o}C tissue culture incubator. The plate no longer needs to be on top of hot water bottle. Return hot water bottle to incubator until next passage.

24. After 2-4 days, organoids will begin to grow quickly, and media will change color. Change media at this point.
25. Typically, organoids are ready to be split again 3-8 days post-passaging. Organoids should be split if any of the following are true:
    a. Organoids are very dense in Matrigel
    b. Organoids have grown very large (>20% the diameter of a 50 µL Matrigel dome)
    c. Matrigel has become very “squishy” and no longer appears firm.

    If organoids are kept too long in culture without passaging, they will begin to die in culture, and portions of organoid will appear dark (especially when viewed with phase-contrast).

Protocol for Freezing Mouse Organoids

Notes:
Typically, we split 1 well of a 24-well plate into 2-4 cryovials, depending on density.

You will need:

- Ice bucket with ice
- 15 mL Falcon tubes on ice
- P1000 and P200 Pipetmen and sterile tips
- Fire-polished pipette or needle w/ syringe
- Cell freezing chamber or 2 Styrofoam tube racks
- (optional, if passaging) Matrigel aliquot on ice
- Splitting Media (+++) on ice
- Refrigerated 15/50 mL tube centrifuge
- Sterile glass pipettes and aspirator
- Recovery Cell Culture Freezing Medium
- (optional, if passaging) Mouse Complete Feeding Media

Procedure:

1. Follow the steps for harvesting organoids and breaking them up detailed in steps 1-14 in the "Protocol for Passaging Mouse Organoids" on page 13.
2. If planning to passage and freeze organoids, divide resuspended organoids into separate 15 mL Falcon tubes, one containing organoids to be split and one containing organoids to be frozen. Set aside organoids to be passaged on ice.
3. Spin organoids to be frozen at 850 rpm (145 RCF) for 5 minutes at 4^{o}C.
4. Carefully aspirate off as much media as possible.
   Get as much media as possible with aspirator and then carefully remove the rest with a P200 Pipetman.
5. Resuspend organoids in 500 µL Recovery Cell Culture Freezing Medium per intended cryovial, and pipette organoids into cryovials.
   Typically, 1 well of a 24 well plate is frozen into 2-4 cryovials, depending on density.
6. Move cryovials into isopropanol cell freezing chamber, and screw lid onto top. (Don't screw lid on too far or chamber will be difficult to open after freezing!)
   a. Alternatively, move cryovials into a 15 mL Falcon Tube Styrofoam rack, cover with a second Styrofoam tube rack, and tape Styrofoam racks together.
7. Incubate freezing chamber with cells at -80^{o}C for 24 hours before transferring cryovials to liquid nitrogen storage.
8. If a portion of organoids was set aside for splitting, proceed with step 16 in the "Protocol for Passaging Mouse Organoids" on page 14.

Protocol for Thawing Mouse Organoids

Notes:
It is important to have 10.5 µM (3.38 µg/mL) Rho Kinase Inhibitor in the Mouse Complete Feeding Media when organoids are thawed to help cells cope with the stress of thawing.

You will need:

- 24- or 48- well tissue culture plate
- 37°C water bath
- 37°C tissue culture incubator
- Ice bucket with ice
- 15 mL Falcon tube on ice
- P1000 and P200 Pipetman and tips
- Refrigerated 15/50 mL tube centrifuge
- Hot-water bottle
- Splitting Media (+++) on ice
- Matrigel aliquot on ice
- Glass aspirator pipettes
- Mouse Complete Feeding Media
- Rho Kinase Inhibitor

Procedure:

1. Place an aliquot of Matrigel on ice.
2. Place Mouse Complete Feeding Media in a 37°C water bath.
3. Prepare a 15 mL Falcon Tube with 10 mL Splitting Media (+++) and place on ice.
4. Remove cryovial containing organoids from liquid nitrogen freezer and thaw quickly in a 37°C water bath.
5. Pipette thawed cells into 15 mL Falcon Tube with Splitting Media (+++).
6. Spin organoids at 850 rpm (145 RCF) for 5 minutes at 4°C.
7. Carefully aspirate off as much media as possible.
   Get as much media as possible with aspirator and then carefully remove the rest with a P200 Pipetman.
9. Resuspend organoid pellet in Matrigel and plate and feed as described in steps 18-23 of the "Protocol for Passaging Mouse Organoids" on page 14.
    a. Depending on size of pellet, make 1-3 wells of organoids. When in doubt, make 1 well. If organoids are too sparse, they will have difficulty recovering from thaw.
    b. Be sure to add Rho Kinase Inhibitor to the Mouse Complete Feeding Media
10. Depending on how quickly the organoids grow, change media 2-4 days after thawing. Once organoids have formed and begun to grow, Rho Kinase Inhibitor is no longer needed in the Mouse Complete Feeding Media.

Protocols for Establishing Organoid Cultures from Normal Human Pancreatic Tissue

Notes:
This protocol describes methods to establish cultures of normal organoids from human pancreatic specimens. These specimens can be obtained either from adjacent normal tissue from surgical resection of pancreatic neoplasms or from islet transplantation programs (ITPs). In the first case, normal pancreatic tissue is taken from the histologically normal pancreas tissue adjacent to neoplastic tissues. In the second case, two different types of specimens can be obtained by ITPs: (i) a suspension of exocrine cell aggregates that are the leftovers of pancreas digestion after human islet purification; or (ii) an unprocessed biopsy.

To avoid the processing of a sample contaminated by neoplastic cells, histological confirmation of normal pancreas is needed when the starting material derives from a resected specimen. To establish tumor organoid cultures from pancreatic cancer specimens see the “Protocol for Establishing Organoid Cultures from Human Pancreas Tumors” on page 24. All human experiments are approved by the IRB of Cold Spring Harbor Laboratory, and written informed consent from the donors for research use of tissue is obtained prior to acquisition of the specimen.

A. Preparation of Solutions for Normal Human Organoid Preparation

All the solutions are prepared before sample processing. Human Splitting Media (++Primocin) and Human Wash Media can be prepared in advance and stored for up to 1 month before use. Human Normal Feeding Media and Human Tumor Feeding Media should be prepared no more than 2 weeks prior to use, due to the instability of some media components.

Human Splitting Media (++ Primocin)

Reagent	Stock Concentration	Volume	Final Concentration
Advanced DMEM/F-12		500 mL	
1M HEPES	100X (1 M)	5 mL	1X (10 mM)
GlutaMAX Supplement	100X	5 mL	1X
Primocin (Invivogen)	400X	1.25 mL	1X

Human Wash Media (keep on ice)

Reagent	Stock Concentration	Volume	Final Concentration
Advanced DMEM/F-12		500 mL	
1M HEPES	100X (1 M)	5 mL	1X
GlutaMAX Supplement	100X	5 mL	1X
Primocin (Invivogen)	400X	1.25 mL	1X
FBS	100%	12.5 mL	2.5% (v/v)

Human Digestion Media

Reagent	Stock Concentration	Volume / Mass	Final Concentration
Human Splitting Media (++Primocin)		20 mL	
Collagenase II *		100 mg	5 mg/mL
Dispase		20 mg	1 mg/mL
FBS **	100%	0.5 mL	2.50%
Soybean Trypsin Inhibitor **	10 mg/mL in PBS	2 mL	1 mg/mL

* Note that human samples are digested in Collagenase II, unlike murine samples, which are digested in Collagenase XI.
** The FBS and Soybean Trypsin Inhibitor have the same function, which is quenching the activity of tryptic enzymes released by acinar cells. Therefore use one or the other.

Human Normal Feeding Media (Pre-warm to 37^{o}C)

Reagent	Stock Concentration	Volume	Final Concentration
Splitting Media (+++)		76 mL	
A83-01*	1000X (0.5 mM)	200 µL	500 nM
mEGF*	1000X (50 µg/mL)	200 µL	50 ng/mL
mNoggin	1000X (100 µg/mL)	200 µL	100 ng/mL
hFGF10*	1000X (100 µg/mL)	200 µL	100 ng/mL
Gastrin I*	1000X (10 µM)	200 µL	0.01 µM
N-acetylcysteine	400X (500 mM)	500 µL	1.25 mM
Nicotinammide	100X (1M)	2 mL	10 mM
B-27 Supplement	50X	4 mL	1X
R-Spondin I-conditioned media	10X	20 mL	1X
Wnt3a-conditioned media	2X	100 mL	1X (50% v/v)
Prostaglandin E2 (PGE2)	1000X (1M)	200 µL	1X (1 µM)
Y-27632**	1000X (10.5 mM)	200 µL	10.5 µM

* 1000X working stocks of A83-01, mEGF, FGF-10, and Gastrin I are prepared by diluting 10,000X frozen stocks – see “Solutions and Key Reagents” for more information.
** Y-27632 (Rho Kinase Inhibitor) is only necessary when organoids are first prepared, when organoids are thawed, or when organoids are dissociated to single cells.
*** We have also tested human normal organoid growth in media with 20% R-spondin1-conditioned media, 30% Wnt3a-conditioned media, and the addition of Forskolin to a final concentration of 1 µM. This formulation also works for human normal organoid culture.

B. Isolation of Human Pancreatic Normal Organoid Cultures from Digested Pancreas from an Islet Cell Transplant Center

This protocol describes methods to establish normal organoid cultures starting from a suspension of exocrine cell aggregates obtained after human islet purification from collagenase-digested pancreas. The cell suspension is usually shipped on ice in a solution of University of Wisconsin solution (UW solution).

You will need:

37°C water bath	Human Normal Feeding Media
Ice bucket with ice	Sterile 5, 10, and 25 mL pipettes
Petri dish (non-tissue culture treated)	15 mL Falcon tubes
Refrigerated 15/50 mL tube centrifuge	Sterile glass pipettes and aspirator
Pipetmen (p10, 20, 1000) and sterile pipette tips	TryplE Express
Rho kinase inhibitor (Y-27632, 10.5 mM stock)	37°C rocking or rotating incubator
Timer	DNAseI (10 mg/mL stock)
Human Wash Media	Pre-warmed hot water bottle
Matrigel	Pre-warmed 24 well culture plate
37°C tissue culture incubator	

Procedure:

1. Upon delivery of the specimen, place the tube containing the cell suspension on ice.
2. Place Human Normal Feeding Media at 37°C to warm.
3. Take a few microliters from the tube and pipette them into a Petri dish.
4. Using an inverted microscope, inspect the specimen in the Petri dish. Check if ductal fragments are visible. If pancreatic ducts are visible, proceed with duct picking as described in the “Protocol for Isolation of Organoids from Normal or PanIN Mouse Pancreas” on page 5, step 20. Then proceed with step 13 of the current protocol.
5. If duct picking is not possible because of the extensive acinar cell contamination, proceed with short trypsinization described in steps 6 to 13 below.
6. Take 10-15 mL from the cell suspension and move into a 15 mL Falcon tube on ice.
7. Centrifuge tube at 200 RCF for 5 min at 4°C
8. Carefully remove the supernatant without disturbing the pellet.
9. Resuspend the pellet into 1.5 mL of TryplE Express containing 1.5 µL 10.5 mM Rho Kinase Inhibitor by pipetting up and down with a p1000.
10. Incubate at 37°C with rocking, rotating, or agitation for a maximum of 15 minutes.
11. At the end of the incubation, check for the presence of cell clumping. If cell clumping is present, add 15 µL of 10 mg/mL DNAse I, and pipette up and down a few times with a p1000 to mix.
12. Stop trypsin digestion by placing the tube on ice and the filling it with Human Wash Media.
13. Pellet digested cells or picked ducts by centrifugation at 200 RCF for 5 min at 4°C.
14. Carefully remove as much media as possible.
15. While keeping the tube of cells on ice, carefully resuspend the pellet in ice-cold Matrigel, while avoiding making bubbles.
    *The amount of Matrigel depends on the pellet size.

16. Plate 50 µL Matrigel domes into a pre-warmed 24 well plate on top of a pre-warmed hot water bottle.
17. Place the plate into a 37^{o}C tissue culture incubator until Matrigel solidifies (typically 5-15 minutes).
18. Add Rho Kinase Inhibitor to your Human Normal Feeding Media as described in the Human Normal Feeding Media recipe on page 19. You will need 500 µL of media per well plated.
19. Add 500 µL of pre-warmed Human Normal Feeding Media supplemented with Rho Kinase inhibitor to each well.
20. Return organoids to 37^{o}C tissue culture incubator.

C. Isolation of Human Pancreatic Adjacent Normal Organoid Cultures from a Resected Specimen

When both normal and neoplastic tissues are available for the establishment of organoid cultures, the uninvolved normal pancreatic tissue will be processed first to avoid tissue autolysis. All the procedures are performed under sterile conditions by using sterile tools and by operating in a biological hood. Different tools should be used for the processing of normal and tumor specimen. Before starting sample processing, ensure that all reagents and supplies needed are ready.

Note that adjacent normal tissue is unlikely to be truly "normal," and that not all tissue a pathologist has described as "normal" behaves like normal tissue when made into an organoid.

You will need:

37°C water bath	Human Normal Feeding Media
Ice bucket with ice	Histology cassettes
Pencil to mark histology cassettes	Petri dishes (non-tissue culture treated)
Sterile scalpels	Sterile forceps
Plastic Jar for neutral buffered formalin	10% Neutral Buffered Formalin (NBF)
Sterile 5, 10, and 25 mL pipettes	50 and 15 mL Falcon Tubes
Human Wash Media	Refrigerated 15/50 mL tube centrifuge
Pipetmen (p20, p200, p1000) and sterile tips	Human Digestion Media
ACK (Ammonium-Chloride-Potassium) Lysis Buffer	DNAse I (10 mg/mL stock)
Sterile glass pipettes and aspirator	TryplE Express
Matrigel	Pre-warmed hot water bottle
Pre-warmed 24 well culture plate	Rho kinase inhibitor (Y-27632, 10.5 mM stock)
37°C tissue culture incubator	

Procedure:

Resected tissue is usually delivered into a 50 mL Falcon tube containing Human Splitting Media (++Primocin) described on page 18. The specimen should be shipped on ice and must be processed as soon as possible to avoid pancreas autolysis. Except for digestions, all procedures are performed on ice.

1. Upon delivery of the specimen, place the tube containing specimen on ice.
2. Place Human Normal Feeding Media at 37°C to warm.
3. Record all the information available for the specimen, including: nosography, hospital, sample identifier, date, and the names of the people processing the sample.
4. Label cassettes for histology with pencil.
5. Prepare Human Digestion Media as described on page 19, and sterile filter it.
6. Transfer the specimen to a Petri dish.
7. Examine the tissue macroscopically to ensure that white hard nodules indicative of potential neoplastic components are not present.
8. Carefully aspirate off the media used for transport.
9. Using a sterile scalpel and sterile forceps, cut the specimen along the biggest diameter into two pieces.
10. Place one piece into a pre-labeled histology cassette, and then place the cassette into a plastic jar containing 10% NBF for fixation.

11. Mince the remaining specimen into small fragments (1 mm^3 or less) using sterile scalpels.
    a. If there is fat present, try to dissect it away from the specimen.
12. Transfer specimen to 15 mL Falcon tube containing 10 mL Human Wash Media.
13. Optional: If there is fat present, let the specimen settle to the bottom of the tube for 1 minute, and aspirate the fat off the top of the liquid.
14. Centrifuge tube at 200 RCF for 5 minutes at 4°C.
15. If no noticeable blood is present, proceed to step 16. Otherwise, if noticeable blood is visible, perform red blood cell lysis as follows:
    a. Carefully aspirate the supernatant from the pellet.
    b. Resuspend the pellet in 5 mL of ACK lysis buffer and place on ice for 5 min.
    c. Centrifuge the tube at 200 RCF for 5 min at 4°C.
16. Carefully aspirate the supernatant from the pellet.
17. Resuspend the pellet in 10 mL Human Digestion Media.
18. Incubate at 37°C with agitation for a maximum of 2 hours.
19. After one hour of digestion time, check digestion by examining tube in microscope.
    a. Check for the presence of cell clumping, which is likely due to the release of DNA from damaged cells.
    b. If cell clumping is present, add 80 µL of a 10 mg/mL DNAse I Stock Solution (final concentration 0.1 mg/mL) to the digestion.
    c. If cells are well digested, and large fragments are broken up, stop the digestion by placing the tube on ice and filling it with ice-cold Human Wash Media.
    d. Otherwise, continue 37°C incubation for up to 2 hours total incubation time.
20. Centrifuge tube at 200 RCF for 5 minutes at 4°C.
21. Wash twice with Human Wash Media, being careful not to disturb the pellet when removing the supernatant.
22. Resuspend the pellet in 1-3 mL of TryplE Express by pipetting up and down few times with a p1000. To avoid cell clumping, add 10-30 µL of a 10 mg/mL DNAse I Stock Solution (final concentration 0.1 mg/mL) to the digestion and pipet up and down with the p1000 a few times.
23. Incubate at 37°C with agitation for 15 min.
24. Stop the trypsinization by placing the tube on ice and filling it with ice-cold Human Wash Media.
25. Centrifuge tube at 200 RCF for 5 minutes at 4°C
26. Carefully remove as much media as possible.
27. While keeping the tube on ice, carefully resuspend the pellet in ice-cold Matrigel*, taking care to avoid making bubbles.
    *The amount of Matrigel to resuspend in depends on the pellet size.
28. Plate 50 µL Matrigel domes into a pre-warmed 24 well plate on top of a pre-warmed hot water bottle.
29. Place the plate into a 37°C tissue culture incubator until Matrigel solidifies (typically 5-15 minutes).
30. Add Rho Kinase Inhibitor to your Human Normal Feeding Media as described on page 19. You will need 500 µL of media per well plated.
31. Add 500 µL of pre-warmed Human Normal Feeding Media supplemented with Rho Kinase to each well.

Protocol for Establishing Organoid Cultures from Human Pancreas Tumors

Notes:
This protocol describes methods to establish tumor organoid cultures from human pancreatic cancer specimens. These specimens are obtained from surgical resections of pancreatic neoplasms. Histological confirmation of pancreatic ductal adenocarcinoma is needed when starting with this material. All human experiments are approved by the IRB of Cold Spring Harbor Laboratory, and written informed consent from the donors for research use of tissue is obtained prior to acquisition of the specimen. All the procedures should be performed under sterile conditions by using sterile tools and by operating in a biological hood. Before starting sample processing, ensure that all reagents and supplies needed are ready.

The Human Tumor Feeding Media is almost identical to the Human Normal Feeding Media described on page 19, except that PGE2 is omitted.

A. Preparation of Solutions for Normal Human Organoid Preparation

Human Splitting Media (++Primocin)

Reagent	Stock Concentration	Volume	Final Concentration
Advanced DMEM/F-12		500 mL	
1M HEPES	100X (1 M)	5 mL	1X (10 mM)
GlutaMAX Supplement	100X	5 mL	1X
Primocin (Invivogen)	400X	1.25 mL	1X

Human Wash Media (keep on ice)

Reagent	Stock Concentration	Volume	Final Concentration
Advanced DMEM/F-12		500 mL	
1M HEPES	100X (1 M)	5 mL	1X
GlutaMAX Supplement	100X	5 mL	1X
Primocin (Invivogen)	400X	1.25 mL	1X
FBS	100%	12.5 mL	2.5% (v/v)

Human Digestion Media

Reagent	Stock Concentration	Volume / Mass	Final Concentration
Human Splitting Media (++Primocin)		20 mL	
Collagenase II *		100 mg	5 mg/mL
Dispase		20 mg	1 mg/mL
FBS **	100%	0.5 mL	2.50%
Soybean Trypsin Inhibitor **	10 mg/mL in PBS	2 mL	1 mg/mL

* Note that human samples are digested in Collagenase II, unlike murine samples, which are digested in Collagenase XI.
** The FBS and Soybean Trypsin Inhibitor have same function, which is quenching the activity of tryptic enzymes released by acinar cells. Therefore use one or the other.

Human Digestion Media – Alternative Recipe

Reagent	Stock Concentration	Volume / Mass	Final Concentration
Human Tumor Feeding Media*		20 mL	
Collagenase II **		100 mg	5 mg/mL
Dispase		20 mg	1 mg/mL

* Since Human Tumor Feeding Media is expensive to produce, but is no longer suitable for organoid culture after 2 weeks, this recipe makes use of left-over, expired Human Tumor Feeding Media. Leftover Human Tumor Feeding Media can be used for up to 6 months for this purpose. Note that this media contains FBS, due to the presence of the Wnt3a-conditioned media.
** Note that human samples are digested in Collagenase II, unlike murine samples, which are digested in Collagenase XI.

Human Tumor Feeding Media (Pre-warm to 37^{o}C)

Reagent	Stock Concentration	Volume	Final Concentration
Splitting Media (+++)		76 mL	
A83-01*	1000X (0.5 mM)	200 µL	500 nM
mEGF*	1000X (50 µg/mL)	200 µL	50 ng/mL
mNoggin	1000X (100 µg/mL)	200 µL	100 ng/mL
hFGF10*	1000X (100 µg/mL)	200 µL	100 ng/mL
Gastrin I*	1000X (10 µM)	200 µL	0.01 µM
N-acetylcysteine	400X (500 mM)	500 µL	1.25 mM
Nicotinammide	100X (1M)	2 mL	10 mM
B-27 Supplement	50X	4 mL	1X
R-Spondin I-conditioned media	10X	20 mL	1X
Wnt3a-conditioned media	2X	100 mL	1X (50% v/v)
Y-27632**	1000X (10.5 mM)	200 µL	10.5 µM

* 1000X working stocks of A83-01, mEGF, FGF-10, and Gastrin I are prepared by diluting 10,000X frozen stocks – see “Solutions and Key Reagents” for more information.
** Y-27632 (Rho Kinase Inhibitor) is only necessary when organoids are first prepared, when organoids are thawed, or when organoids are dissociated to single cells.

B. Isolation of Human Tumor or Metastatic Organoids from a Resected Specimen

You will need:

- 37°C water bath
- Ice bucket with ice
- Pencil to mark histology cassettes
- Sterile scalpels
- Plastic Jar for neutral buffered formalin
- Sterile 5, 10, and 25 mL pipettes
- Human Splitting Media (++Primocin)
- Pipetmen (p20, p200, p1000) and sterile tips
- ACK (Ammonium-Chloride-Potassium) Lysis Buffer
- Sterile glass pipettes and aspirator
- Matrigel
- Pre-warmed 24 well culture plate
- 37°C tissue culture incubator
- (Optional) Cryovials
- (Optional) -80°C freezer
- (Optional) CAF Media
- (Optional) 6 well culture dish
- Human Tumor Feeding Media
- Histology cassettes
- Petri dishes (non-tissue culture treated)
- Sterile forceps
- 10% Neutral Buffered Formalin (NBF)
- 50 and 15 mL Falcon Tubes
- Refrigerated 15/50 mL tube centrifuge
- Timer
- Human Digestion Media
- DNAse I (10 mg/mL stock)
- TryplE Express
- Pre-warmed hot water bottle
- Rho kinase inhibitor (Y-27632, 10.5 mM stock)
- (Optional) Recovery Cell Culture Freezing Medium
- (Optional) Cell freezing chamber
- (Optional) Liquid nitrogen cryofreezer

(Optional) CAF Media

450 mL RPMI 1640 Medium with L-Glutamine
5 mL 100X Penicillin/Streptomycin
50 mL 100% FBS

Procedure:

Resected tissue is usually delivered into a 50 mL Falcon tube containing Human Splitting Media (++Primocin) as described on page 24. The specimen should be shipped on ice (not dry ice!) and can be shipped overnight (to arrive in the lab the day after surgery). Except for digestions, all procedures should be performed on ice.

1. Upon delivery of the specimen, place the tube containing specimen on ice.
2. Place Human Tumor Feeding Media at 37°C to warm.
3. Prepare Human Digestion Media following the recipe on page 24 or 25, and sterile filter it.
4. Record sample information: nosography, hospital, deidentified sample number, date and names of the people processing the sample.
5. Label cassettes for histology with pencil.
6. Transfer the specimen into a Petri dish and record the size, shape, and gross morphological characteristics.
7. Carefully aspirate off the media used for transport.
8. Using a sterile scalpel, cut the specimen along the biggest diameter.
9. Cut a piece from the center of the specimen, place the specimen in a pre-labeled histology cassette, and then place the cassette into a plastic jar containing 10% NBF for fixation.
10. Mince the remaining specimen into small fragments (1 mm^3 or less) using sterile scalpels.
    a. Pancreatic tumors are highly fibrotic, and therefore, the tissue is usually hard.
    b. If there is any fat present, try to dissect it away from the specimen.
11. Transfer specimen to 15 mL Falcon tube containing 10 mL Human Wash Media or Human Splitting Media (++Primocin).

12. Optional: If there is fat present, let the specimen settle to the bottom of the tube for 1 minute, and aspirate the fat off the top of the liquid.
13. Centrifuge tube at 200-400 RCF for 5 minutes at 4°C.
14. Carefully remove supernatant.
15. Resuspend pellet in 8 mL Human Digestion Media.
16. Incubate the digestion at 37°C with agitation for an initial digestion of 1 hour.
17. After one hour of digestion time, look at tube to see how digested the fragments are at the macroscopic level and inspect tube in microscope to see how digested tissue is at the microscopic level. Check for the presence of cell clumping, which is likely due to the release of DNA from damaged cells.
    a. If everything is digested into submacroscopic size pieces, proceed to step 18.
    b. Otherwise, incubate digestion at 37°C with agitation for an additional hour and check again. If large chunks are still visible, check again 2 hours later (after 4 hours total digestion time). Keep digesting until the big chunks are well broken up. It is fine if there are large clumps of cells visible in microscope as long as the macroscopic chunks are digested.
    c. If cell clumping is present, up to 80 µL of a 10 mg/mL DNAse I Stock Solution (final concentration 0.1 mg/mL) can be added to the digestion tube.
    d. Optional – after 1-2 hours of digestion time, the tube can be allowed to settle for a few minutes, and the floating cells can be separated from the sunken cells to generate 2 separate organoid lines, a quick digesting and a slow digesting line. These lines would then be recombined into a single line at the first passage.
18. Centrifuge the digest tube at 200-400 RCF for 5 minutes at 4°C.
19. If there is no visible blood present, proceed to step 20. In the unlikely event that the tumor is well vascularized (more likely with FNA/FNB than resected tumor) and a noticeable amount of blood is visible, perform red blood cell lysis:
    a. Carefully remove the supernatant without disturbing the pellet.
    b. Resuspend the pellet in 5 mL ACK lysis buffer and place on ice for 5 min.
    c. Centrifuge the tube at 200-400 RCF for 5 min at 4°C.
20. Carefully remove the supernatant without disturbing the pellet.
21. Optional: If the tissue needs further digestion, resuspend the pellet in 1-3 mL of TrypLE Express. Add 10-30 µL 10 mg/mL DNaseI Solution to prevent clumping. Pipette up and down with a P1000 to mix.
22. Incubate at 37°C with agitation for 2-10 minutes.
23. Centrifuge the digest tube at 200-400 RCF for 5 minutes at 4°C.
24. Wash the digest tube twice with Splitting Media (++Primocin) or with Wash Media, being careful not to disturb the pellet when removing the supernatant.
25. Optional: If pellet is quite large, you can freeze some of the digest:
    a. Separate a portion of digest to a new 15 mL Falcon tube.
    b. Centrifuge at 200-400 RCF for 5 min at 4°C.
    c. Carefully remove the supernatant.
    d. Resuspend pellet in Recovery Cell Culture Freezing Medium
    e. Transfer to one or more cryovials.
    f. Freeze overnight in cell freezing chamber at -80°C, and transfer to cryofreezer.
26. Optional: To isolate cancer associated fibroblasts:
    a. Separate a portion of digest to a new 15 mL Falcon tube.
    b. Centrifuge at 200-400 RCF for 5 min at 4°C.

c. Carefully remove the supernatant.
d. Resuspend pellet in CAF Media.
e. Transfer to 1 well of a 6 well culture plate.
f. Place in 37^{o}C tissue culture incubator.
g. At future passages, very short trypsin incubations will help dissociate fibroblasts while keeping cancer cells attached. This method may be used to enrich for fibroblasts.

27. Centrifuge the digest tube at 200-400 RCF for 5 min at 4^{o}C, and carefully remove as much media as possible.
28. While keeping the tube on ice, carefully resuspend the pellet in ice-cold Matrigel, taking care to avoid making bubbles.
    a. The amount of Matrigel to resuspend in depends on the pellet size. A good pellet could easily yield 12 wells.
29. Plate 50 µL Matrigel domes into a pre-warmed 24 well plate on top of a pre-warmed hot water bottle.
30. Place the plate into a 37^{o}C tissue culture incubator until Matrigel solidifies (typically 5-15 minutes).
31. Add Rho Kinase Inhibitor to Human Tumor Feeding Media as described on page 25. You will need 500 µL of media per well plated.
32. Add 500 µL of pre-warmed Human Tumor Feeding Media supplemented with Rho Kinase inhibitor to each well.
33. Return plate to tissue culture incubator. Plate no longer needs to be on hot water bottle, which can also be returned to the tissue culture incubator.

Protocols for Passaging, Freezing, and Thawing Human Organoid Cultures

Notes:
The protocols for passaging, freezing, and thawing human organoid cultures are almost identical to those described for mouse organoids (see “Protocol for Passaging Mouse Organoids” on page 13, “Protocol for Freezing Mouse Organoids” on page 16, and “Protocol for Thawing Mouse Organoids” on page 17).

There are, however, special considerations to keep in mind when working with human cultures:

- While mouse organoids are split using Splitting Media (+++), human organoids can be split using either Splitting Media (+++) or Human Splitting Media (++Primocin).
- Due to the heterogeneity observed in human cancers, many organoid morphologies can be observed.
    - If the human organoids appear cystic, similar to mouse organoids, splitting can be easily performed using fire-polished pipettes.
    - If the human organoids appear to be filled with cells, alternative split protocols may be better:
        - Option 1: Combine the fire-polished pipette method with a short incubation in TrypLE Express (5-10 min at 37^{o}C). Use DNAse I Stock Solution to a final concentration of 10 µg/mL if clumping is observed. Include Rho Kinase Inhibitor in the Human Normal or Tumor Feeding Media.
        - Option 2: Use the protocol to generate single cells as described on page 36. Include Rho Kinase Inhibitor in the Human Normal or Tumor Feeding Media.
        - Option 3: Incubate the organoids in Corning Cell Dissociation Solution at 4^{o}C for 20-30 minutes to free the cells from the Matrigel, and then do a short incubation in TrypLE Express (5-10 min at 37^{o}C). Use DNAse I Stock Solution to a final concentration of 10 µg/mL if clumping is observed. Include Rho Kinase Inhibitor in the Human Normal or Tumor Feeding Media.
- Human organoids generally do not grow as fast as mouse organoids. The splitting ratio used should generally be 1:2 or 1:3.
- While human tumor organoids do not appear to have a passaging limit, human normal organoids will stop propagating after approximately 20 passages.
- When freezing human organoids, freeze one confluent 24 well into 1-2 cryovials for optimal recovery.

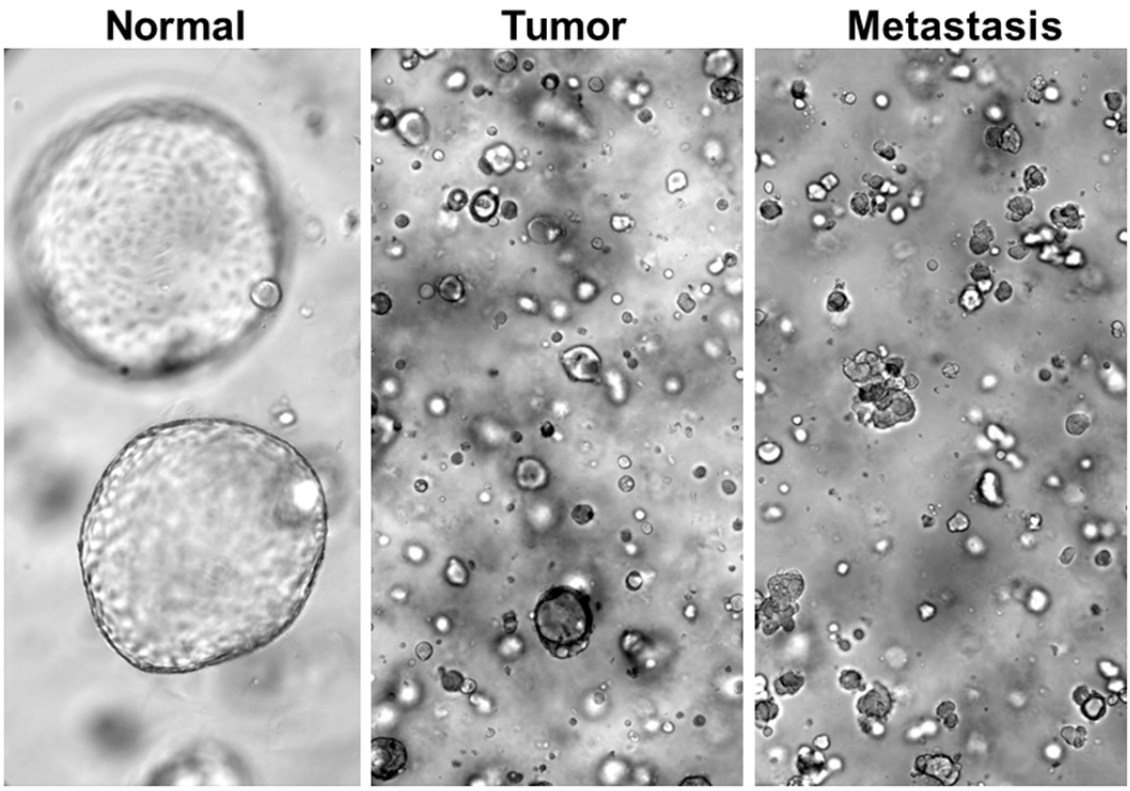

Figure 4. Human normal, tumor, and metastatic organoids.
Human normal organoids appear as spherical cysts, similar to murine organoids. Human tumor and metastatic organoids can take a variety of morphologies, including filled spheres.

Protocol for Developing a 2D Cell Line from a Human Organoid Culture

Notes:

- The 2D cell line is developed by allowing the outgrowth of organoids that naturally attach to the plastic at the bottom of a well of organoids in Matrigel.
- Not all human organoid cultures form cell lines.
- A couple of passages after you initiate an organoid culture (once you have lost the fibroblast contamination), check your plate to see if some of the cells in your culture have sunk through the Matrigel and attached to the plastic. These cells that have attached to the tissue culture plastic will be the start of your 2D cell line. You will generate your 2D cell line by removing the organoids and Matrigel from the well, and letting your cells outgrow in the same well.
- The more wells with cancer cells stuck to the bottom that you can start with, the easier it is to establish a 2D cell line.

You will need:

37°C water bath	Organoid plate with cancer cells attached to bottom of well
Ice bucket with ice	Human 2D Media (prewarmed to 37°C)
Pipetmen (p1000) and sterile tips	Human Splitting Media (++Primocin)
37°C tissue culture incubator	15 mL Falcon Tubes
PBS (tissue culture grade)	Trypsin (tissue culture grade)

Media recipes:

Human Splitting Media (++Primocin)

Reagent	Stock Concentration	Volume	Final Concentration
Advanced DMEM/F-12		500 mL	
1M HEPES	100X (1 M)	5 mL	1X (10 mM)
GlutaMax	100X	5 mL	1X
Primocin	400X	1.25 mL	1X

Human 2D Media (prewarmed to 37°C)

Reagent	Stock Concentration	Volume	Final Concentration
RPMI 1640 Medium with L-Glutamine		450 mL	
FBS	100%	50	10%
Penicillin/Streptomycin	100X	5 mL	1X

* With L-glutamine - manufacturer # 10-040-CV.

Procedure:

1. Use a glass aspirator pipette to remove the media from each well to be harvested
2. Pipette 500 µL cold Human Splitting Media (++Primocin) onto each Matrigel dome to be harvested.
3. Pipette up and down gently to break up the dome and solubilize the organoids. Do not pipette too harshly to ensure cancer cells attached to the plastic stay attached.
4. Transfer the media with the Matrigel and organoids to a 15 mL Falcon Tube.
5. Use 500 µL cold Human Splitting Media (++Primocin) to gently rinse each well.
6. Add 500 µL warm Human 2D Media to each well.
7. Place plate back in tissue culture incubator (37°C, 5% CO_2, 20% O_2).

8. If desired, complete the organoid passaging protocol to passage the organoids you isolated in the Falcon Tube.
9. Monitor the growth of your 2D cancer cells over time. You should see the cells forming colonies within the well of the 24- or 48-well plate, and those colonies growing in size over the next 1-2 weeks. However, not all human organoid lines are capable of proliferating in 2D culture conditions.
10. Once colonies of cancer cells are covering the majority of the well(s) it is time to passage the cells into a larger dish:
    a. Use PBS and trypsin to passage cells like any other 2D cell line.
    b. Trypsinization time should be determined empirically for each line. Many pancreas cancer cell lines require longer trypsinization times than fibroblast cells.
    c. If you are culturing more than 1 well of the same line, the wells should all be combined during this passaging step.
    d. The size of the tissue culture dish you passage into should be determined based on how many wells of 2D cells, with a goal of not diluting your cells too much. Typically, splitting the cells into 1-2 wells of a 6 well plate (or 1-2, 35 mm tissue culture dishes) is a good next step, since these have approximately 5x the area as one well of a 24 well plate.

Protocol for Harvesting RNA from Organoid Cultures

Notes:
Typically, 1 confluent well of organoids in a 24 well plate yields ~3-5 µg total RNA.
We usually pool 3-6 wells of organoids from a 24 well plate.

You will need:

Eppendorf tubes	Ice bucket with ice
24- or 48- well plate with organoids	Glass pipettes and aspirator
TRIzol Reagent	P1000 and P200 Pipetmen and tips
(optional) Liquid nitrogen	(optional) -80^{o}C freezer
(for TRIzol protocol) Chloroform	(for TRIzol protocol) Isopropanol
(for TRIzol protocol) Ethanol	(for TRIzol protocol) Glycogen

Procedure:

1. Place Eppendorf tubes to receive RNA on ice to chill.
2. For the wells of organoids to be harvested, carefully aspirate media away from Matrigel domes.
3. Pipette 1 mL TRIzol Reagent on top of first Matrigel dome to be harvested.
4. Pipette up and down approximately 10 times, until Matrigel begins to dissolve into TRIzol, and bits of Matrigel dome are no longer visible clinging to the plate.
5. Pipette mixture of TRIzol, cells, and Matrigel onto next well of organoids to be harvested.
6. Repeat steps 4 and 5 until all wells of organoids to be harvested together have been combined into the TRIzol mixture.
7. Transfer the mixture of TRIzol, cells, and Matrigel into cold Eppendorf tube.
8. (Optional) Flash freeze Eppendorf tubes in liquid nitrogen and store at -80^{o}C until ready to prepare RNA.
9. RNA can now be prepared using your favorite protocol.
10. If using the TRIzol protocol, we suggest the following modifications:
    a. Make sure to measure the amount of aqueous phase and add an equal volume of isopropanol - Often the aqueous phase volume is higher than the 0.5 mL the TRIzol protocol assumes, and a 1:1 ratio of aqueous phase:isopropanol is necessary for RNA to pellet.
    b. We also recommend using glycogen as recommended in the TRIzol protocol to help pellet the RNA.
11. If highly pure RNA is needed for RNA-sequencing or other sensitive downstream applications, we recommend purifying RNA using the “TRIzol Plus” protocol which comes with the Pure Link RNA Mini Kit (Life Technologies 12183018A). Briefly, thaw RNA, add chloroform, and spin all as described in the TRIzol protocol. Remove aqueous phase to a new tube, and add 1 volume 70% ethanol as described in the Pure Link RNA Mini Kit protocol. Follow the Binding, Washing, and Elution steps described in the Pure Link RNA Mini Kit protocol.

Protocol for Harvesting Protein from Organoid Cultures

You will need:

LoBind 1.5 mL Tubes	15 mL Falcon tubes
24- or 48- well plate with confluent organoids	Ice bucket with ice
Protease Inhibitor Tablets	Glass pipettes and aspirator
DPBS	Pipettman (P1000) and tips
Refrigerated 15/50 mL tube centrifuge and microfuge	PhosSTOP Tablets
Refrigerated microfuge	Lysis Buffer (TNET or SDS Lysis)
Gel loading tips	Liquid nitrogen
-80^{o}C freezer	

Lysis Buffer Recipes:

TNET

1% Triton X 100
150 mM NaCl
5 mM EDTA
50 mM Tris, pH 7.5

SDS LYSIS BUFFER	Recipe to Make 10 mL:
10 mM Tris pH 7.5	0.1 mL of 1M Tris pH 7.5
1% SDS	1 mL of 10% SDS
	8.9 mL milliQ water

Procedure:

1. Ensure that organoid wells are at least 80%-90% confluent before beginning, since this is crucial to getting a good cell pellet.
2. Add protease inhibitor tablet and PhosSTOP tablet to DPBS Solution to generate DPBS-PPI Solution. Keep this on ice.
3. Add protease inhibitor tablet and PhosSTOP tablet to lysis buffer (either TNET or SDS LYSIS BUFFER) to generate either TNET-PPI or SDS LYSIS-PPI. If using TNET-PPI, place this solution on ice.
4. Aspirate media from the organoid domes.
5. Add 500 µL of ice-cold PBS-PPI to each dome and pipette up and down to manually disrupt the Matrigel.
6. Pool 2 organoid mounds from a 24 well plate (1 mL total volume) into 1 Lo-Bind 1.5 mL tube, or transfer up to 8 wells of a 24 well plate into a 15 mL tube, in 10 mL total volume of cold DPBS-PPI.
7. Centrifuge cells:
    a. For 1.5 mL tubes: 3000 RCF for 3 minutes in a chilled microfuge.
    b. For 15 mL tubes: max speed for 5 minutes in a chilled 15/50 mL tube centrifuge.
8. If organoids were harvested at 80-90% confluency, the organoids should sediment cleanly below the Matrigel layer and should appear as a white pellet below the gel.
9. Using a gel loading tip, carefully aspirate off the DPBS solution and the Matrigel, leaving behind the cell pellet. To get a clean pellet with minimal Matrigel, you must aspirate on the upper edge of the cell pellet and sacrifice a little bit of the cell pellet.
10. Wash the pellet 2x in ice-cold DPBS-PPI Solution as described above.

a. If cells are in a 15 mL Falcon tube, use 2nd wash to transfer to a 1.5 mL LoBind tube.

11. For TNET Lysis:
    a. Resuspend the pellet in 100 µL of TNET-PPI and if not already in an Eppendorf tube, transfer to a LoBind Eppendorf tube.
    b. Incubate on ice for 10 minutes.
    c. Pass the lysate three times through a 26 Gauge or insulin needle with a 1 mL syringe to shear chromatin.
    d. Centrifuge the lysate 10 minutes at max speed in a microfuge at 4°C.
    e. Transfer supernatant to a new LoBind Eppendorf tube.
12. For SDS-PPI Lysis:
    a. Heat SDS-PPI Buffer to 100°C.
    b. Add 100 µL boiling SDS-PPI Buffer to the pellet of cells.
    c. Incubate cells at 100°C for 5 min and allow to cool to room temperature.
    d. Pass the lysate three times through a 26 Gauge or insulin needle with a 1 mL syringe to shear chromatin.
13. Protein concentration can be determined by Lowry Method.
14. Flash freeze samples in liquid nitrogen and store samples at -80°C until needed.

Note: As an alternative to cold PBS washes, Corning Cell Recovery Solution (Corning #354253) can be used to isolate cells from the Matrigel. Cells can then be washed in DPBS and resuspended in Lysis Buffer as described in steps 10-14 above.

Protocol for Generating Single Cells from Organoid Cultures

Note: This protocol works well for both mouse and human organoids

You will need:
[For single cell prep]

50 mL Falcon Tube	Dispase
Digital scale	5, 10, 25 mL pipettes
Splitting Media (+++)	Glass pipettes, aspirator, and filter free tips
24- or 48- well plate with organoids	Pipettman (P2, P20, P200, 1000) and tips
Timer	Ice bucket with ice
LoBind 1.5 mL and 5 mL Tubes	Refrigerated 15/50 mL tube centrifuge and microfuge
DNAse solution (10 mg/mL stock)	37^{o}C rocking/rotating incubator
Refrigerated microfuge	Microscope
TrypLE Express	(optional) Cell counting chambers and solution

[For culturing]

Human or Mouse Complete Feeding Media	Rho kinase inhibitor (Y-27632, 10.5 mM stock)
Matrigel	Pre-warmed hot water bottle
Pre-warmed 24 well culture plate	37^{o}C tissue culture incubator

Procedure:

1. Measure out Dispase in a 50 mL Falcon tube
    a. You will need approximately 1.8 mg per well of organoid you are harvesting
    b. Measure out at least 25 mg.
2. Bring tube of Dispase to the tissue culture hood.
3. Add cold or room temperature Splitting Media (+++) to the Dispase to bring it to a concentration of 2 mg/mL. Invert tube to make sure dispase is resuspended in the solution.
    a. Technically, the Dispase Solution is not sterile, because the Dispase was measured outside the tissue culture hood. We have not had problems with sterility. However, if you experience problems, you could try sterile filtering the Dispase solution. However, the concentration of Dispase may need to be adjusted if it is filtered to account for Dispase that may stick to the filter.
4. Remove media from organoid wells to be harvested.
5. Add 500 µL Dispase Solution to each well to be harvested.
6. Pipette up and down with a P1000 to break up each Matrigel dome.
7. Incubate plate in tissue culture incubator (37^{o}C, 5% CO_2, 20% O_2) for 10 minutes.
8. Use pipette to move the Dispase/Organoid mixture to a Protein LoBind 1.5 mL or 5 mL tube
    a. Depending on the number of wells to be harvested.
    b. Up to 2 wells per 1.5 mL tube or up to 8 wells per 5 mL tube.
9. Use 0.5 mL (for the 1.5 mL tube) or 1 mL (for the 5 mL tube) of Dispase Solution to wash the wells and combine with the Dispase/Organoid mixture in the LoBind tube.
10. Add 1 – 5 µL 10 mg/mL DNase solution to the Dispase/Organoid mixture.

    a. The purpose of the DNase solution is to prevent DNA that is released from dying cells from causing cells to clump.

11. Incubate at 37^{o}C with rocking/rotation for 10 minutes.
12. Spin tube 200-400 RCF for 5 minutes.
13. Remove most of the supernatant, but leave a bit to ensure that you don't lose the pellet.
14. Wash the cells once in 1 mL TrypLE Express and spin again.
    a. If cells are in 5 mL tube, use this step to move cells to 1.5 mL tube.
15. Remove most of the supernatant, but leave a bit to ensure that you don't lose the pellet.
16. Add 1 mL TrypLE Express and 1 µL 10 mg/mL DNase solution.
17. Pipette up and down gently 10-20 times to resuspend the cells.
18. Incubate at 37^{o}C with rocking/rotation for 5 minutes.
19. Gently pipette up and down 10-20 times to help dissociate cells.
20. Look at tube in microscope to see if cells are dissociated.
21. If cells are not dissociated, repeat steps 18-20 until cells are dissociated.
    a. This has taken up to 35 minutes for some organoid lines, with no apparent impact on viability.
22. Spin tube 200-400 RCF for 5 minutes.
23. Remove most of the supernatant, but leave a bit to ensure that you don't lose the pellet.
24. Wash cells 3 times in 1 mL Splitting Media (+++).
25. Option #1: To Count the Cells
    a. Resuspend the cells in Splitting Media (+++) and use a cell counter to count cells
        i. A typical, relatively confluent organoid well in a 24 well plate has 100,000-300,000 cells, so for counting, I usually resuspend the cells in 50-100 µL per well harvested.
    b. Based on cell counts, set aside a set number of cells for replating
        i. Typically, cells are replated at a concentration of 25,000 – 50,000 cells per well of a 24 well plate.
    c. Spin tube with cells to be plated 200-400 RCF for 5 minutes.
26. Option #2: To Virally Transduce Cells
    a. Single cells can be transduced by Lentivirus or Retrovirus.
    b. Aliquot 20,000 – 200,000 cells per transduction.
    c. Refer to the Lentiviral Infection protocol on page 41, step 14.
27. Option #3: To Use Cells for Flow Cytometry
    a. The cells are ready for flow – spin down once more, wash once in your flow buffer of choice, and resuspend cells in your flow cytometry staining buffer
28. Option #4: To Replate Cells
    a. Spin cells to be plated 200-400 RCF for 5 minutes.
    b. Carefully remove all of the supernatant (use a manual pipetteman for the final 100 µL).
    c. Resuspend the cells in 50 µL Matrigel per well to plate.
    d. Spot Matrigel domes onto a prewarmed 24 well plate.
    e. Let the domes set by keeping the plate in the tissue culture incubator 15 minutes.
    f. While domes set, add Rho Kinase Inhibitor to your organoid media. You will need 0.5 mL per plated well.
    g. Add 500 µL prewarmed organoid media (*with Rho Kinase Inhibitor) to each well.

Transfection to Make Lentivirus / Lentiviral Infection of Organoids

Notes:
Protocol from Daniel Öhlund 10/31/13
For this protocol, your lentiviral vector must be compatible with the second generation packaging system (psPAX1 and pMD2.G). Examples of vectors compatible with the second generation system are pLKO.1 and pLVX.
Check with your university on the rules and regulations surrounding lentiviral production.

You will need:
[For transfection and virus production]

Lentiviral Vector (uses 2nd generation packaging)	psPAX1 plasmid
pMD2.G plasmid	DMEM
X-tremeGene9 Transfection Reagent	Pipettman (P20, P200, 1000) and sterile tips
Sterile 1.5 mL tubes	293T cells
DMEM, 10% FBS, 1% Penicillin/Streptomycin	PBS
Trypsin	Cell counting chambers and dye
6 cm tissue culture dish	37°C tissue culture incubator
Glass pipettes and aspirator	5, 10, 25 mL pipettes
Refrigerated 15/50 mL centrifuge	Splitting Media (+++)
LentiX Concentrator	-80°C Freezer

[For infection of organoids]

24- or 48-well plate of organoids to be infected	Glass pipettes and aspirator
Pipettman (P20, P200, 1000) and sterile tips	5, 10, 25 mL pipettes
Splitting Media (+++)	Ice bucket with ice
15 mL Falcon tube	Fire-polished glass pipettes
Refrigerated 15/50 mL tube centrifuge	Timer
37°C rocking/rotating incubator	TrypLE Express
Lentivirus aliquot	DMEM, 5% FBS, 1% Penicillin/Streptomycin
48 well organoid culture plate	250X Polybrene Stock
37°C tissue culture incubator	Non-refrigerated 15/50 mL tube centrifuge
Mouse, Human Normal, or Human Tumor Feeding Media	Rho kinase inhibitor (Y-27632, 10.5 mM stock)
Matrigel	Pre-warmed hot water bottle
Pre-warmed 24 well culture plate	(optional) Antibiotic for selection

4 mg/mL (1000X) Polybrene Solution (recipe for 40 mL)

0.16 g Polybrene
H2O to fill to 40 mL

Polybrene is also known as Hexadimethrine bromide
Polybrene is also known as 1,5-Dimethyl-1,5-diazaundecamethylene polymethobromide
Sterile filter through 0.2 µM filter, aliquot, and store at -20°C.
Freeze thaws are ok.

Procedure:
Transfection of 293T cells to produce virus

1. Prepare transfection mix inside a 1.5 mL tube in a tissue culture hood.
    a. ____ µL DMEM (serum free)
    b. 1 µg Target lentiviral vector (*2nd generation packaging compatible)
    c. 0.75 µg psPAX1
    d. 0.25 µg pMD2.G
    e. Total volume = 94 uL
2. Add 6 µL X-tremeGene9 Transfection Reagent, and pipette up and down gently with a P1000 to mix.
3. Incubate for 20 min at room temperature.
4. Trypsinize a plate of 293T cells, and make a suspension of $2.5x10^6$ cells in 5 mL media (DMEM, 10% FBS, 1% Penicillin/Streptomycin).
5. Mix cells with the transfection mix.
6. Plate cells and transfection mix in a 6 cm tissue culture dish and incubate overnight in 37°C tissue culture incubator.
7. 24 hours after plating, change media to 5 mL fresh media.
8. 24 hours after changing the media, harvest the virus-containing supernatant and move to 15 mL Falcon tube.
9. Filter virus through 0.45 µM filter into clean 15 mL Falcon tube.
10. For 2D cells: virus can be used in a 1:5 dilution
11. For Organoids, Concentrate the virus:
    a. Add Lenti-X Concentrator to the viral supernatant. Determine the volume of Lenti-X Concentrator to add by taking the total volume of viral supernatant and dividing by 3.
    b. Incubate virus and concentrator at 4°C overnight.
    c. Centrifuge virus and concentrator at 1500 RCF for 45 min at 4°C.
    d. Aspirate supernatant away from pellet.
    e. Resuspend viral pellet in 1 mL Splitting Media (+++)
    f. Aliquot into 0.25 mL aliquots.
    g. Store viral aliquots in – 80°C freezer.
    h. 1 x 0.25 mL aliquot should be used for 1 infection.

Infection of Organoids

1. Thaw lentivirus aliquot on ice.
2. Aspirate media away from Matrigel domes.
3. Add 500 µL ice-cold Splitting Media (+++) into the well
4. Pipette up and down to break organoids
5. Transfer organoids into 15 mL Falcon tube with 10 mL ice-cold Splitting Media (+++).
6. Centrifuge at 850 rpm (145 RCF) for 5 min at 4°C.
7. Remove 8~8.5 mL of media and leave 1.5~2 mL media.
8. Pipette up and down with a fire-polished glass pipette 10 times.
9. Add Splitting Media (+++) up to 10 mL.

10. Centrifuge at 850 rpm (145 RCF) for 5 min at 4°C.
11. Carefully remove supernatant.
12. Add 1 ml TrypLE Express and incubate at 37°C with gentle agitation for 5 min.
13. Quench TrypLE by adding 9 ml DMEM, 5% FBS, 1% Penicillin/Streptomycin.
14. Centrifuge at 150-200 RCF for 5 min at 4°C.
15. Carefully remove supernatant.
16. Resuspend cell pellet with 250 µL virus with 1 µL 250X Polybrene Stock
    a. Dilute the 1000X Polybrene Stock in DPBS to get a 250X stock.
17. Transfer the cell suspension with virus into a single well of 48 well culture plate
    a. Be sure to use the special plates for culturing organoids.
18. Centrifuge at 600 RCF (~1700 rpm) for 1 hour at room temperature.
19. Incubate the plate at 37°C in a tissue culture incubator for 1-6 hours.
20. Resuspend cells with 1 mL Splitting Media (+++) and transfer cells to 15 ml Falcon tube.
21. Add 9ml ice-cold Splitting Media (+++).
22. Centrifuge at 850 rpm (113 RCF) for 5 min at 4°C.
23. Remove the supernatant.
24. Resuspend with 100~ 200 µL Matrigel and plate into 24 well plate (50 µL / well) on a hot water bottle.
25. Incubate 37°C tissue culture incubator to allow Matrigel to harden 15 min.
26. Add Rho Kinase Inhibitor to Mouse, Human Normal, or Human Tumor Feeding Media. You will need 500 µL Feeding Media per well plated.
27. Add 500 µL of Mouse, Human Normal, or Human Tumor Feeding Media (with Rho Kinase inhibitor added) to each well.
28. Return plate to 37°C tissue culture incubator.
29. Select with antibiotics 2 days after infection.
    a. Optimal concentration for antibiotic must be determined empirically.

Additional Reagent Information

Matrigel

Need 125 µL for organoid isolation and 25 µL per small (48-well plate) well or 50 µL per large (24-well plate) well when passaging
Matrigel should be in -20 freezer or on ice at all times.
Matrigel begins to harden at temperatures above 0^{o}C.
Allow enough time for your Matrigel aliquot to thaw on ice before you need it: an 800 µL aliquot of frozen Matrigel takes ~ 1 hour and 15 minutes to thaw on ice, and a full vial takes ~8 hours.
To aliquot a full vial, thaw on ice overnight, and aliquot into pre-chilled tubes using pre-chilled tips.

10 mg/mL DNase I

Prepare in advance at 10 mg/mL in sterile DPBS, and aliquot and store at -20^{o}C.
Used for organoid isolation

10% Neutral Buffered Formalin (NBF)

The Tuveson Lab buys NBF.
Store at room temperature.
Used for organoid isolation.

TrypLE

TrypLE is more gentle than ordinary trypsin.
Store at room temperature.
Used for T or M organoid isolation, human isolation, and single cell preps.

Fire-polished pipettes

Used for passaging or freezing organoids.
Fire-polished pipettes are glass pipettes whose ends have been constricted by rotating the pipettes in a fire.
To make a fire-polished pipette, set up a Bunsen burner inside of a tissue culture hood. Rotate a glass tissue culture pipette quickly while holding the very end of the pipette in the flame of the Bunsen burner. The goal is to narrow the opening of the glass pipette to approximately half of the diameter of the starting pipette.

Hot-water bottle

At all times, we keep a hot water bottle in the 37^{o}C tissue culture incubator. The bottle serves as a warming plate to keep 24-well/48-well tissue culture plates at 37^{o}C while making Matrigel domes of organoids. Since Matrigel hardens at 37^{o}C, the hot water bottle enables the tissue culture plate to stay warm while Matrigel/organoid domes are spotted, ensuring that the domes begin to solidify immediately after they are spotted. This reduces the risk of Matrigel domes collapsing after spotting.
To make:

In a tissue culture hood, fill a T-75 75cm^{2} tissue culture flask to top with sterile water.
Add a few drops of a water bath antifungal agent.
Close up flask, and seal cap with parafilm.
Prepare in advance and store in the tissue culture 37^{o}C incubator.

FEEDING MEDIA FACTORS

Advanced DMEM/F-12

Used for Splitting Media (+++), Mouse Complete Feeding Media, Human Normal Complete Feeding Media, Human Tumor Complete Feeding Media
Store at 4°C.

100X Penicillin/Streptomycin (Thermo-Fisher)

Used for Mouse Wash Media, Mouse Digestion Media, Splitting Media (+++), Mouse Complete Feeding Media, Human Normal Complete Feeding Media, Human Tumor Complete Feeding Media
Aliquot and store at -20°C.

100X (1M) HEPES Buffer (Thermo-Fisher)

Used for Splitting Media (+++), Mouse Complete Feeding Media, Human Normal Complete Feeding Media, Human Tumor Complete Feeding Media
Store at 4°C.

100X GlutaMAX Solution (Thermo-Fisher)

Used for Splitting Media (+++), Mouse Complete Feeding Media, Human Normal Complete Feeding Media, Human Tumor Complete Feeding Media
Store at 4°C.

A83-01

50,000X A83-01 Concentrated Stock (25 mM, 10.52 mg/mL)

Resuspend 10 mg in 950 µL DMSO.
Store 20 µL aliquots at -20°C

1000X A83-01 Working Stock (0.5 mM, 0.21 mg/mL)

Mix 5 µL 50,000X A83-01 Concentrated Stock with 245 µL DMSO.
Store 20 µL aliquots at -20°C.
Once thawed, keep working aliquot at 4°C.

mEGF

10,000X mEGF Concentrated Stock (500 µg/mL)

Resuspend 1 mg in 2 mL 0.1% BSA/DPBS.
Store 200 µL and 5 µL aliquots at -20°C.

1000X mEGF Working Stock (50 µg/mL)

Mix 5 µL 10,000X mEGF Concentrated Stock with 45 µL Splitting Media (+++).
Store at 4°C.

FGF-10

10,000X FGF-10 Concentrated Stock (1 mg/mL)

Resuspend 250 µg in 250 µL 0.1% BSA/DPBS.
Store 50 µL aliquots at -20°C. Aliquots are good for 3 months.

1000X FGF-10 Working Stock (0.1 mg/mL)

Mix 50 µL 10,000X FGF-10 Concentrated Stock with 450 µL 0.1% BSA/DPBS.
Store 20 µL aliquots at -20°C.
Once thawed keep working aliquot at 4°C.

Gastrin I

10,000X Gastrin I Concentrated Stock (100 µM, 0.21 mg/mL)

Resuspend 0.1 mg in 480 µL DPBS.
Store 20 µL aliquots at -20°C.

1000X Gastrin I Working Stock (10 µM, 0.021 mg/mL)

Mix 20 µL 10,000X Gastrin I Concentrated Stock with 180 µL Splitting Media (+++).
Store at 4°C.

mNoggin

1000X mNoggin Working Stock (100 µg/mL)

Resuspend 20 µg in 200 µL 0.1% BSA/DPBS.
Store 20 µL aliquots at -20°C. Aliquots are good for 3 months.
Once thawed, keep working aliquot at 4°C.

N-Acetylcysteine

400X N-Acetylcysteine Working Stock (500 mM, 81.6 mg/mL)

Resuspend 5 g in 61.2 mL sterile H2O and filter through 0.22 µM filter.
Store 1 mL and 50 µL aliquots at -20°C.
Once thawed, keep working aliquot at 4°C.

Nicotinamide

100X Nicotinamide Working Stock (1M, 122 mg/mL)

Resuspend 1.22 g in 10 mL DPBS.
Store 500 µL aliquots at -20°C.
Once thawed, keep working aliquot at 4°C.

50X B27 Supplement

Purchase and store 400 µL aliquots at -20°C
Once thawed, keep working aliquot at 4°C.

R-Spondin1-Conditioned Media

See “Production of Rspondin1-conditioned Media” on page 49 for more information.
In the Tuveson Lab, we use RSPO1-conditioned media at 10X.
Note, that in our hands, recombinant R-Spondin1 did not work.

Y-27632 (Rho Kinase Inhibitor)

1000X Y-27632 Working Stock (10.5 mM, 3.38 mg/mL)

Resuspend 5 mg in 1480 µL sterile H2O.
Store 25 µL aliquots at -20°C.
Once thawed, keep working aliquot at 4°C.

PGE2

10,000X Concentrated Stock

Make at 10M in DMSO

1000X PGE2 Working Stock (1M)

Dilute concentrated stock 1:10 in DMSO

Forskolin

10,000X Concentrated Stock

Make at 10M in DMSO

1000X Forskolin Working Stock (1M)

Dilute concentrated stock 1:10 in DMSO

Wnt3a- conditioned media

Cells engineered to produce Wnt3a can be obtained from ATCC (L Wnt-3A, ATCC #CRL-2647).
See “Production of Wnt-3a-conditioned Media” on page 47 for more information.

OTHER REAGENTS

DMEM For Mouse Wash Media, Mouse Digestion Media
100% FBS For Mouse Wash Media, Mouse Digestion Media
Collagenase Crude Type XI For Mouse Digestion Media
Collagenase Type II For Human Digestion Media
Dispase II For Mouse and Human Digestion Media
DMSO (sterile) For dissolving media components
DPBS (no Ca^{+2}, no Mg^{+2}, sterile) For dissolving media components
Albumin Solution from Bovine Serum, 30% in DPBS (30% BSA, sterile)
For dissolving media components (make 0.1% BSA/PBS solution using 30% BSA and DPBS)
Sterile H2O For dissolving media components
Recovery Cell Culture Freezing Medium For freezing organoids
Store 3 mL aliquots at -20°C.
Thaw before use and refreeze unused media.

More Information About Feeding Media Components

MEDIA COMPONENT	INFO ABOUT COMPONENT
A83-01	Inhibits ALK, leading to TGF Beta Inhibition
mEGF	Growth Factor
FGF-10	Growth Factor, Activates NOTCH pathway
Gastrin I	Hormone, stimulates pancreatic acinar cells to secrete digestive enzymes, pancreatic growth factor
mNoggin	Inhibits BMP4, chordin, follistatin, leading to TGF Beta Inhibition
N-acetylcysteine	Antioxidant
Nicotinamide	B vitamin; inhibits poly(ADP-ribose) polymerases (PARP-1), promotes endocrine lineage
B27 supplement	Leads to retinoic acid activation; contains steroids and antioxidants
R-spondin I	Activates Wnt pathway, will drive Myc
Y-27632	Rho kinase inhibitor; helps cells cope with stress
PGE2	Agonist of Prostoglandin E2 receptor. Activates the Wnt pathway
Wnt3a	Activates Wnt pathway, will drive Myc

Production of Wnt-3a-conditioned Media

Notes:

- The Tuveson Laboratory uses a special Wnt3a-expressing line that has a zeocin selectable marker, using the protocol below.
- A similar Wnt3a-expressing cell line, with a G418-selectable marker can be purchased from ATCC (L Wnt-3A, ATCC CRL-2647).
- ***If using the ATCC cell line to produce Wnt3a-conditioned media, follow the protocol below, but substitute the ATCC-recommended concentration of G418 for the zeocin.
- Cells can be used for around 10 passages to harvest more batches of conditioned medium.
- Wnt3a can only be produced in medium with FBS.
- Wnt activity in Wnt3a conditioned media can be tested using the TOPflash assay

You will need:

Culture Media	100 mg/mL Zeocin Selection Reagent
Sterile trypsin	Sterile PBS
175 cm2 tissue culture flasks	Serological pipettes (5, 10, and 25 mL)
L-Wnt3a Cells	Pipetman (p10, 20, 1000)
15 cm tissue culture dishes	Sterile Pipette Tips with Filters
Sterile 0.2 µM filter	15 and 50 mL Falcon tubes
15/50 mL tube centrifuge	Sterile glass aspirator pipettes

Culture Media

500 mL DMEM
60 ml 100% FBS
5 ml Penicillin/Streptomycin

100 mg/mL Zeocin Selection Reagent

Use 1.25 µL per ml Culture Medium

Procedure:

1. Thaw a frozen vial of L-wnt3a cells and transfer cells into a 15 mL Falcon tube with 9.5 mL Culture Media.
2. Centrifuge cells at 133 RCF for 5 minutes and remove media.
3. Resuspend cells 1 mL Culture Media and seed cells in a 175 cm^2 culture flask containing 50 mL Culture Medium.
4. Culture cells until the flask is confluent (typically 2-4 days).
5. Split the confluent flask by washing once with 25 mL PBS, incubating with 2 mL trypsin, and quenching with 18 mL media.
6. Centrifuge cells at 133 RCF for 5 minutes and remove media.
7. Resuspend cells in 6 mL Culture Media and transfer 1 mL each to 6 x 175 cm^2 culture flasks each containing 50 mL Culture Media. Add 62.5 µL Zeocin to only one of the flasks (the “Selection Flask”), while leaving the other 5 flasks (the “Conditioning Flasks”) free of Zeocin.
8. When the “Conditioning Flasks” are confluent (after 2-4 days):
    a. Carefully pour off the media from the cells and wash the cells with 25 mL PBS.
    b. Trypsinize the cells using 2 mL trypsin.
    c. Quench the trypsin with 18 mL Culture Media without Zeocin
    d. Pool all the cells in a 1 L sterile glass bottle. Add additional Culture Media without Zeocin to bring the total volume to 600 mL.

e. Plate 20 mL of cells onto 15 cm culture dishes, making a total of 30 dishes.
f. Incubate cells for one week in the tissue culture incubator.
g. After one week, use a serological pipette to remove the media from the cells into transfer to 50 mL Falcon tubes.
h. Centrifuge the media for 5 minutes at 300 RCF to pellet floating cells.
i. Carefully pour the supernatants into a 0.2 μM sterile filter attached to a sterile bottle and filter the media.
j. Aliquot filtered media and either freeze and store at -20^{o}C or store at 4^{o}C.

9. When the “Selection Flask” is confluent, repeat steps 5-8.

Production of Rspondin1-conditioned Media

Notes:

Rspo1-expressing cells can be obtained from the laboratory of Calvin Kuo by establishing an MTA. Information can be found on his laboratory website: http://kuolab.stanford.edu/

Cells can be used for 10-12 passages to harvest batches of conditioned medium.

You will need:

Culture Media	100 mg/mL Zeocin Selection Reagent
Sterile trypsin	Sterile PBS
175 cm2 tissue culture flasks	Serological pipettes (5, 10, and 25 mL)
L-Wnt3a Cells	Pipetman (p10, 20, 1000)
15 cm tissue culture dishes	Sterile Pipette Tips with Filters
Sterile 0.2 µM filter	15 and 50 mL Falcon tubes
15/50 mL tube centrifuge	Sterile glass aspirator pipettes

Culture Media
- 500 mL DMEM
- 60 ml 100% FBS
- 5 ml Penicillin/Streptomycin

100 mg/mL Zeocin Zeocin Selection Reagent
- Use 3 µL per ml Culture Medium (final concentration 300 µg/mL) for selection

Splitting Media (+++) – Used to Generate the Conditioned Media
- 500 ml Advanced DMEM/F-12
- 5 mL Penicillin/Streptomycin
- 5 mL 1 M Hepes
- 5 ml GlutaMAX Supplement

Protocol:

1. Thaw a frozen vial of 293T-HA-Rspo1-Fc cells, and transfer cells into a 15 mL Falcon tube with 9.5 mL Culture Media.
2. Centrifuge cells at 133 RCF for 5 minutes and remove media.
3. Resuspend cells 1 mL Culture Medium and seed cells in a 175 cm^2 culture flask containing 50 mL Culture Medium and 150 µL 100 mg/mL Zeocin Selection Reagent.
4. Culture cells until the flask is confluent (typically 2-4 days).
5. Split the confluent flask by washing once with 25 mL PBS, incubating with 2 mL trypsin, and quenching with 18 mL media.
6. Centrifuge cells at 133 RCF for 5 minutes and remove media.
7. When the “Conditioning Flasks” are confluent (after 2-3 days):
    a. Carefully pour off the media from the cells and wash the cells 2x with 25 mL PBS.
    b. Add 50 mL Splitting Media (+++) to each flask.
    c. Incubate cells for one week in the tissue culture incubator.
    d. After one week, carefully decant the media from the cells into transfer to 50 mL Falcon tubes. (Cells may detach easily.)
    e. Centrifuge the media for 5 minutes at 300 RCF to pellet floating cells.
    f. Carefully pour the supernatants into a 0.2 µM sterile filter attached to a sterile bottle and filter the media.
    g. Aliquot filtered media and either freeze and store at -20^{o}C or store at 4^{o}C.
8. When the “Selection Flask” is confluent, repeat steps 5-8.

Pancreas Orthotopic Transplantation

Protocol from Dannielle Engle

Notes:
Please see the following reference for additional guidance and images:

Kim MP, Evans DB, Wang H, Abbruzzese JL, Fleming JB, Gallick GE. Generation of orthotopic and heterotopic human pancreatic cancer xenografts in immunodeficient mice. Nat Protocols. 2009;4(11):1670-80.

Suggestions/Cautions:

- Practice this procedure with your animal facility veterinarian on euthanized mice before attempting this surgery on live animals, especially suturing and wound clipping.
- In the beginning, your procedures will take longer and will benefit from the use of a heated surface. If you can trim down the procedure time to 10 minutes, the heating pad is not necessary.
- Using high cell numbers can increase the risk of cell leakage. If cells leak into the abdominal cavity, a secondary tumor at the wound site on the peritoneum as well as development of malignant ascites is possible and will require the mice to be euthanized earlier.

You Will Need:
[Cell Preparation]

Ice bucket with ice	Sterile glass pipettes and aspirator
Pipetmen (P20, P200, P1000) and sterile tips	24- or 48-well plate of organoids to harvest
Dipase	Splitting Media (+++)
5 mL LoBind tube	37°C rocking/rotating incubator
Refrigerated 15/50 mL centrifuge	TrypLE
10 mg/mL DNAse	Phenol Red Free Splitting Media (PRF+++)
Cell counting chambers and dye	Matrigel

[Surgery]

Hot bead sterilizer	Surgery cards
Isoflurane/Oxygen mixture	Cells to be transplanted
Shea scissors*	Graefe Forceps*
Dissolvable Sutures, Taper needle (Ethicon Vicryl or PDS II)	0.3-0.5 cc insulin syringes
Wound Clipper (Reflex)	Wound Clips (Reflex 7mm)
Matrigel	Tissue Glue
Analgesic (Ketoprofen)	Eye Gel
Topical Antibiotic	Isoflurane
Betadine	Sterile Sponges
Sterile Cotton Tips	Trypan Blue or GI Spot

* Tool available from Fine Science Tools

Procedure:

1. Prepare cells (always prepare more than needed - 20% excess):
    a. Begin with organoids grown to confluence and dissociate them into single cells.
    b. Remove media and harvest Matrigel mounds in 1–4 mg/ml Dispase in Splitting Media (+++) into a 5 mL LoBind tube.
        i. Harvest 6 wells with 1 mL of Dispase in a MW24.

        ii. Dispase concentration depends on the activty of current lot of Dispase. Begin with 1 mg/mL, but if this is insufficient to dissolve Matrigel in 30 min, increase in 1 mg/ml increments.
    c. Incubate tube rocking at 37^{o}C for 15-30 minutes.
    d. Spin cells (200 RCF, 5 minutes) and remove sup
        i. Determine whether there is residual Matrigel.
        ii. If there is, add fresh Dispase and dissociate for another 30 minutes as above.
        iii. If not, then proceed.
    e. Add TrypLE (2–3 mL) with 10 µL 10 mg/mL DNase.
    f. Incubate at 37^{o}C for 5-30 minutes with occasional vortexing
    g. Monitor thereafter every 5 minutes until single cell dissociate looks sufficient
        i. Add more DNAse if see clumping (5 µL / 5 mL).
    h. Spin cells as above and remove sup
    i. Wash cells in Phenol Red Free Splitting Media (PRF+++), spin, and remove sup
    j. Resuspend cells in PRF+++, and count cells.
    k. Make working dilution of cells in 50% Matrigel/PRF+++
        i. Some people use the more concentrated form of Matrigel or Matrigel that has not been depleted with growth factors, but growth factor reduced Matrigel also works
    l. Keep cells on ice
2. Turn on the hot bead sterilizer and 37^{o}C Recovery Chamber
3. Begin labeling Surgery Cards
4. Clean and organize work space
5. Perform laparotomy
    a. Sterilize tool tips
    b. Induce the mouse with 4% Isoflurane/Oxygen
    c. Transfer the mouse to nose cone, reduce Isoflurane to 2%
    d. Apply eye gel to mouse
    e. Administer analgesics (5 mg/kg Ketoprofen)
    f. Apply ear code to the mouse
    g. Pluck the hair from the wound site (over the spleen) (~ 3 cm diameter)
        i. Alternatively, remove fur with hair clippers followed by Nair (5 min)
        ii. Make sure to remove all Nair from wound site using water and sponges
    h. Sterilize the area using Betadine + Cotton Tip, three times, beginning in the center and moving outwards
    i. Make a small incision (0.5 – 1.0 cm) in the skin perpendicular to the spleen using Shea scissors
    j. Make a smaller incision through the peritoneum same orientation
    k. Exteriorize the spleen by applying very gentle traction using Graefe forceps
        i. Do not close your forceps all the way, this will cut the spleen in half
    l. The pancreas should be attached to the spleen, avoid handling the pancreas excessively, but if needed pull gently to lay out the pancreas
    m. Do not fully expose the pancreas until ready to proceed so that it stays moist
6. Prepare cell injection
    a. Load an insulin syringe with the appropriate volume of cells (20 – 50 µL)
7. Pancreas injection
    a. If the pancreas appears dry, add a drop of sterile saline
    b. Pull the pancreas up vertically
    c. Make sure to locate the major blood vessels
    d. Insert needle in the tail of the pancreas in between your forceps and push the needle down parallel to the major blood vessel as far as the needle will insert
    e. Pull the needle a very small fraction to either side to insure you are within the parenchyma and not outside the pancreas

f. Press down the plunger and you should be able to see the formation of a clear bubble
    i. You can add 1 µL of Trypan Blue or GI Spot per injection (sterile filtered) to aid in visualizing injections until you are comfortable
g. Slowly retract the needle by pulling it up straight
h. Release the pancreas by gently laying it back down

8. End of procedure
    a. Gently move the spleen and pancreas into the peritoneal cavity
        i. Do not put pressure on the bubble from the injection, use the spleen as a handle for the pancreas
    b. Suture the peritoneum (purse string)
    c. Wound clip the skin
        i. Make sure to tent the skin when wound slipping to prevent snagging the peritoneum
    d. Apply tissue glue
9. Place mouse in the 37^{o}C recovery chamber and allow it to wake up prior to transferring to its cage
10. Annotate the details of each mouse by ear code
    a. Include whether the bubble was intact, or estimate percentage of leakage
    b. Include whether there was bleeding or if you nicked the pancreatic blood vessels, if a hematoma formed
    c. Record whether any mouse took longer than normal to recover and any other notable instances (i.e. if the mouse just ate and its cecum is bloated, it can obscure the pancreas and will require that the cecum be gently exteriorized. This will lengthen procedure time and require a lengthening of the incision).

Orthotopic Transplant Worksheet

Line Name, Passage & Date	Viability	Cell Concentration	Working Concentration	Injection Volume Pancreas

Mouse Ear Code & ID, Cage #	Notes

Product Information

Reagent	Vendor	Catalog #	Quantity
For Organoid Culture:			
Matrigel – Growth Factor Reduced	Corning	356231	10 mL
24 well tissue culture plates	VWR (Must be Greiner BioOne)	82050-892	100 plates per case
48 well tissue culture plates	VWR (Must be Greiner BioOne)	82051-004	100 plates per case
For Organoid Isolation:			
Neutral Buffered Formalin	Fisher Scientific	22-110-869	2.5 Gal
#10 Scalpels	VWR	89176-380	Pack of 10
Biopsy Cassette (with Attached Covers)	VWR	18000-240	500 per case
Petri dishes	VWR	25384-342	500 per case
Square Petri Dishes for Duct Picking	VWR	60872-310	500 per case
Dissection tools (assorted)	Fine Science Tools		
DMEM (High-Glucose)	VWR	45000-304	6 x 500 mL per case
FBS	VWR	97068-085	500 mL
Dispase II	Thermo Fisher	17105041	5 g
Collagenase Crude Type XI	Sigma-Aldrich	C9407-1G	1 g
DNase I (bovine pancreas)	Sigma-Aldrich	D5025-150KU	150 KU
ACK Lysing Buffer	Thermo Fisher	A1049201	100 mL
Soybean Trypsin Inhibitor, powder	Thermo Fisher	17075029	1 g
Primocin	Invivogen	ant-pm-2	1g in 20 mL liquid
Colagenase II	Thermo Fisher	17101-015	1 g
TrypLE Express Enzyme, Phenol Red	Thermo Fisher	12605010	100 mL
For Splitting Media (+++):			
Advanced DMEM/F-12	Thermo Fisher	12634010	500 mL
GlutaMAX 100X	Thermo Fisher	35050061	100 mL
Penicillin/Streptomycin (10,000 U/mL)	Thermo Fisher	15140122	100 mL
Hepes 1 M	Thermo Fisher	15630080	100 mL
For Growth Factor Stock Resuspension:			
DMSO	Sigma-Aldrich	D2650-100ML	100 mL
Dulbecco's PBS, DPBS, no Ca+2 or Mg+2	Thermo Fisher	14190144	500 mL
Albumin solution from bovine serum BS 30%	Sigma-Aldrich	A9576-50ML	50 mL

Reagent	Vendor	Catalog #	Quantity
For Organoid Complete (Feeding) Media:			
N-Acetylcysteine	Sigma-Aldrich	A9165-5G	5 g
Nicotinamide	Sigma-Aldrich	N0636-100G	100 g
mNoggin	Peprotech	250-38	100 µg
EGF Recombinant Mouse Protein	Thermo Fisher	PMG8041	100 µg
Recombinant Human FGF-10	Peprotech	100-26	100 µg
Gastrin I	TOCRIS	3006	1 mg
A83-01 (TGF-β inhibitor)	TOCRIS	2939	10 mg
B27 supplement 50x (serum-free)	Thermo Fisher	17504044	10 mL
Prostaglandin E2 (PGE2)	R&D Systems	2296 / 10	10 mg
Forskolin	R&D Systems	1099/10	10 mg
Corning Polystyrene Tissue Culture Flasks	VWR	89090-950	case of 50
Y-27632 (Rho Kinase inhibitor)	Sigma-Aldrich	Y0503	5 mg
L Wnt-3A Cells	ATCC	CRL-2647	1 vial
Zeocin Selection Reagent	Thermo Fisher	R25001	8 x 1.25 mL
For Freezing, Thawing, and Preparing Single Cell Suspensions:			
Recovery cell culture freezing medium	Thermo Fisher	12648010	50 mL
For Working With Organoids			
Trizol Reagent	Thermo Fisher	15596018	200 mL
Eppendorf Protein LoBind 5 mL Tubes	Sigma-Aldrich	Z768812-100EA	100 per box
Eppendorf Protein LoBind 1.5 mL Tubes	Sigma-Aldrich	Z666505-100EA	100 per box
X-tremeGENE 9 DNA transfection reagent	Sigma-Aldrich	6365779001	400 µL
Corning Cell Recovery Solution	Corning	354253	100 mL
Lenti-X™ Concentrator	Clontech	631231	100 mL
RPMI 1640 Medium with L-Glutamine (10040CV)	Fisher Scientific	MT10040CV	6 x 500 mL
PhosSTOP	Sigma-Aldrich	4906845001	10 tablets
cOmplete Mini, EDTA-free Protease Inhibitor Cocktail	Sigma-Aldrich	11836170001	25 tablets
Hexadimethrine bromide	Sigma-Aldrich	H9268-5G	5 g

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